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Solubility of Acetylene in Lung Tissue as an Error in Cardiac Output Determination with the Acetylene Method

By

R JERNERUS G LUNDIN and L G C F PUGH

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Abstract

JERNERUS R G LUNDIN and L G C F PUGH *Solubility of acetylene in lung tissues as an error in cardiac output determination with the acetylene method* Acta physiol scand 1963 59 1—6 — The solubility of acetylene in lung tissue is measured by a rebreathing technique Enough acetylene is dissolved in the lung tissue to simulate an extra gaseous lung volume between 0 27—0 48 litres This should give an error of 7—12 in cardiac output determination with the acetylene rebreathing method

The interest in indirect methods for cardiac output determinations is steadily increasing and the development of fast electronic gas analysers has provided new possibilities of checking the validity of the classical methods During a series of work experiments we wished to measure cardiac output and for technical reasons we used the acetylene method (GROLLMAN 1932) With one notable exception (ASMUSSEN and NIELSSEN 1953) this method has given values that are too low compared with the direct Fick method dye-dilution and even carbon-dioxide rebreathing methods (HAMILTON 1948 WERKO BERSEUS and LAGERLOF 1949 CHAPMAN *et al* 1950 and DEFARES 1956) The low values obtained by the acetylene method are ascribed mainly to recirculation during the rebreathing period

A factor that seems to have been grossly neglected is the solubility of acetylene or other gases in lung tissues although it was pointed out as early as 1911 when MARKOFF MULLER and ZUNTZ published the first report of an inert gas (N_2O) technique for measuring the blood flow through the lungs of man that this

solubility must be taken into account *The stimulus to the present experiments* was the failure to explain the discrepancy in results between the multiple breath foreign gas method and other methods for cardiac output determinations when using the acetylene method We assumed that the uptake of acetylene in lung tissue could be estimated from a simultaneous determination of the dilution of the nitrogen in the lungs and acetylene in the rebreathing bag when inhaling from the bag a mixture of acetylene and oxygen of known volume and composition

Methods

The experiments were carried out on three subjects The ventilatory function of the lungs was controlled by measuring the increase in nitrogen percentage when the subjects were rebreathing from a rubber bag filled at the outset with about 2 7 litres of oxygen A nitrogen meter was used to follow the nitrogen percentages A complete mixing between the gases in the lung bag system seemed to have been obtained after 3 consecutive inhalations and expirations indicating normal ventilatory properties of the lungs

In the present experiments the bag was filled with about 30 % acetylene in oxygen. The gas volume in the bag could be checked with a sensitive manometer After sampling 50 ml of the gas for analysis the bag contained 2 700 ml After a normal expiration the subject changed over from air breathing to breathing in and out of the bag After 4 breaths which were completed in 5—6 sec a sample of 50 ml was taken into a pre evacuated burette and after another 4 breaths a second sample was taken in the same way after which the rebreathing was stopped The samples were then analysed in a modified Haldane apparatus When the experiment was finished the following values were known initial volume and composition of gas in the bag and the percentages of acetylene oxygen and nitrogen in the lung bag system after 4 and 8 breaths

The gaseous lung volume was calculated according to the following equation

Formula (1)

$$\frac{F_{N_1}}{F_{N_2}}(V_{ln} + 2\ 7)F_{N_2} = 0\ 80 \times V_{ln}$$

where V_l is the gaseous lung volume in litres 2 7 the volume of the bag F_{N_1} and F_{N_2} the nitrogen fractions in the first and second rebreathing samples and 0 80 the normal fraction of nitrogen in alveolar air $\frac{F_{N_1}}{F_{N_2}}$ is a correction factor for the decrease in lung bag volume during the first rebreathing period due to the absorption of oxygen and acetylene We have assumed that this uptake is the same during the first and second rebreathing periods and that the amount of nitrogen given off from blood passing through the lungs during a 5—6-second period is negligible The acetylene dissolving lung tissue volume was calculated according to the following equation

Formula (2)

$$\frac{F_{N_1}}{F_{N_2}}(V_l + 2\ 7)\,F_{C_2H_2} + F_{C_2H_2} \times V_t \times 0\ 760 + (V_{ln} + 2\ 7)$$
$$\left(F_{C_2H_2} - F_{C_2H_2} \times \frac{F_{N_1}}{F_{N_2}}\right) + (F_{C_2H_2} - F_{C_2H_2})\,V_t \times 0\ 760 = F^{bag}_{C_2H_2} \times 2\ 7$$

where V_{ln} is the lung volume determined from the N_2 values and assumed to be the gaseous lung space V_t is the tissue volume into which the C_2H_2 is dissolved and 0 760

Table I

Subject	Lung vol (V_{la})	Tissue vol (V_{ti})
G L	2.80	0.46
	3.12	0.59
	2.77	0.76
	2.82	0.62
R, J	3.27	0.38
	2.52	0.50
	2.62	0.36
	3.03	0.20
G P	3.54	0.36
	2.55	0.68
	2.89	0.37
	2.59	0.99

Volume in litres BTPS
Volume in litres.

is the solubility coefficiency for acetylene in lung tissue (CANDER 1959) $F_{C_2H_2}$ and $F'_{C_2H_2}$ are the fractions of acetylene in the first and second rebreathing samples and $F^{bag}_{C_2H_2}$ is the acetylene fraction in the bag before rebreathing $\frac{F_{N_2}}{F'_{N_2}}$ is a correction factor for the influence of the volume change of the lung bag system on the measured fractional values The first term of the equation represents the amount of gaseous acetylene in the lung bag system at the time of the fourth breath The second term expresses the acetylene dissolved in the lung tissue at the time of the fourth breath The third term represents the amount of acetylene removed by the blood from the gaseous lung space during the first rebreathing period and the fourth term is the amount of acetylene dissolved in lung tissue carried away by the blood during the same period We have assumed that the disappearence rate of both gaseous and dissolved acetylene is the same during the two rebreathing periods All the above terms should add up to the amount of acetylene in the bag before rebreathing starts

Results

The lung volumes determined from the dilution of the lung nitrogen and the acetylene dissolving lung tissue volumes V_{ti} are given in Table I The average V_{ti} amounts for subject G L to 0.63 for G P to 0.60 and for R J to 0.36 litres

Discussion

When KROGH and LINDHARD (1911) introduced their single breath N_2O method for cardiac output determination they also strongly criticised the rebreathing method of MÜLLER *et al* and pointed out that recirculation must

solubility must be taken into account. The stimulus to the present experiments was the failure to explain the discrepancy in results between the multiple breath foreign gas method and other methods for cardiac output determinations when using the acetylene method. We assumed that the uptake of acetylene in lung tissue could be estimated from a simultaneous determination of the dilution of the nitrogen in the lungs and acetylene in the rebreathing bag when inhaling from the bag a mixture of acetylene and oxygen of known volume and composition.

Methods

The experiments were carried out on three subjects. The ventilatory function of the lungs was controlled by measuring the increase in nitrogen percentage when the subjects were rebreathing from a rubber bag filled at the outset with about 2.7 litres of oxygen. A nitrogen meter was used to follow the nitrogen percentages. A complete mixing between the gases in the lung bag system seemed to have been obtained after 3 consecutive inhalations and expirations indicating normal ventilatory properties of the lungs.

In the present experiments the bag was filled with about 30 % acetylene in oxygen. The gas volume in the bag could be checked with a sensitive manometer. After sampling 50 ml of the gas for analysis the bag contained 2 700 ml. After a normal expiration the subject changed over from air breathing to breathing in and out of the bag. After 4 breaths which were completed in 5—6 sec a sample of 50 ml was taken into a pre evacuated burette and after another 4 breaths a second sample was taken in the same way after which the rebreathing was stopped. The samples were then analysed in a modified Haldane apparatus. When the experiment was finished the following values were known: initial volume and composition of gas in the bag and the percentages of acetylene, oxygen and nitrogen in the lung bag system after 4 and 8 breaths.

The gaseous lung volume was calculated according to the following equation.

Formula (1)

$$\frac{F_{N}}{F_{N}}(V_{LN} + 2.7)F_{N_2} = 0.80 \times V_{LN}$$

here V_L is the gaseous lung volume in litres, 2.7 the volume of the bag, F_{N_1} and N_2 the nitrogen fractions in the first and second rebreathing samples and 0.80 the normal fraction of nitrogen in alveolar air. $\frac{F_{N_1}}{F_{N_2}}$ is a correction factor for the decrease in lung bag volume during the first rebreathing period due to the absorption of oxygen and acetylene. We have assumed that this uptake is the same during the first and second rebreathing periods and that the amount of nitrogen given off from blood passing through the lungs during a 5—6-second period is negligible. The acetylene dissolving lung tissue volume was calculated according to the following equation.

Formula (2)

$$\frac{F_{N_1}}{F_{N_2}}(V_{LN} - 2.7)F_{C_2H_2} + F_{C_2H_2} \times V_t \times 0.760 + (V_{LN} + 2.7)$$

$$\left(F_{C_2H_2} - F_{C_2H} \times \frac{F_{N}}{F_{N}}\right) + (F_{C_2H_2} - F_{C_2H})V_t \times 0.760 = F^{bag}_{C_2H_2} \times 2.7$$

where V_L is the lung volume determined from the N_2 values and assumed to be the gaseous lung space, V_t is the tissue volume into which the C_2H_2 is dissolved and 0.760

venous blood CO_2 content of 55 vol % (BARTELS and OPITZ 1958) also shows that a difference in CO_2 elimination between the two rebreathing periods is too small to influence the results significantly

Fluctuations in blood flow through the lungs during the rebreathing period would vitiate the results only if the difference in blood flow between the two rebreathing periods was of the order of several hundred per cent Earlier experiments using the GROLLMAN acetylene method for cardiac output determinations have shown that the pulse rate followed with an electronic pulse counter is constant during the rebreathing which was taken as a proof that no large changes in blood flow take place during rebreathing of this kind The observed acetylene uptaking tissue V_t should thus be real values This means that the major part of the lung tissue is rapidly saturated with acetylene This tissue should then behave as an extra lung space amounting for G L to 0 48 for G P to 0 46 and for R J to 0 27 litres The lower values for R J could perhaps be explained by his smaller size and weight as compared with the other two subjects These values are also of the same order of magnitude as those obtained by CANDER and FORSTER This difference in acetylene and nitrogen lung volume will influence the cardiac output determinations with the Grollman acetylene method The arteriovenous oxygen difference is obtained from the relationship

$$\text{fall in } F_{O_2}/\text{fall in } F_{C_2H_2}$$

during the two rebreathing periods The acetylene however is given off to the blood from a larger volume viz $V_{in} + V_t$ 0 76 than the oxygen V_t and this will delay the fall of the acetylene concentration with a consequent overestimation of the arteriovenous oxygen difference and a subsequent underestimation of the cardiac output For our subjects this error should amount to about 12 % for G L and G P and to 7 % for R J assuming an average gas volume of 4 litres for the lung bag system

The solubility of acetylene or N_2O in pulmonary parenchymal tissue appears to be a factor that has been overlooked since the foreign gas method was introduced by MARKOFF MULLER and ZUNTZ (1911) If this factor is taken into account a better agreement between the foreign gas method and other methods for cardiac output determinations will result

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The Influence of the Dietary Fat on the Lethal Temperature in the Hypothermic Rat

By

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Abstract

HUTTUNEN M and B W JOHANSSON *The influence of the dietary fat on the lethal temperature in the hypothermic rat* Acta physiol scand 1963 59 7—11 — Rats were given a semisynthetic diet containing 30 per cent fat One series received unsaturated fats (corn oil) and another saturated fats (coconut oil) A small series was used as control After about 3 months the animals were cooled and the oesophageal temperature at the last heart beat was registered The hearts from the coconut oil series stopped beating at a significantly higher temperature (12 9 C) than the hearts from the corn oil series (7 2 C) The exact explanation for the difference between the two series is not known but it is suggested that the physical properties of the myocardial cells especially the cell membranes are of importance for the tolerance to hypothermia

The hibernators are among other things characterized by their ability to decrease their body temperature to a few degrees centigrade during hibernation in the winter The factors necessary for hibernation are manifold but details of these are lacking Inborn qualities such as the ability of the cardiovascular and nervous systems to function at temperatures just above 0 C as well as exogenous and endogenous factors are important (JOHANSSON 1960) To these factors belong for example the surrounding temperature inhibition of temperature regulation and hypertrophy of the brown fat

The inborn qualities allow the heart of hibernators to function at a temperature much lower than is possible for other homeothermic animals It is possible that the enzymes are less temperature dependant and that the temperature activity curve of the enzymes from hibernators is broad covering a temperature range from 0 —5 C to about 40 C while the corresponding curve



Fig 1 shows the mean weight (x) and the highest and lowest weights of rats receiving a semisynthetic diet containing 50 per cent saturated fats (coconut oil) and unsaturated fats (corn oil) respectively

for non hibernating homeothermic animals is narrower with a peak around 37° C It is probable that the physical properties of the tissues, especially the cell membranes might also be of importance It is known that the hamster, a hibernator can decrease the saturation of depot fats in a low environmental *temperature while this is not possible for the rat a non hibernator* (FAWCETT and LYMAN 1954) These authors also showed that a diet rich in Newol (iodine value 15) decreased the iodine number while the opposite occurred using a diet rich in peanut oil (iodine value 93)

It is conceivable that the dietary fat changes not only the iodine number and melting point of the depot fat but also of lipids in other parts of the body such as the heart This is supported by the work of HORWITT *et al* (1959) They found that the relation between linoleic acid and oleic acid in erythrocytes was significantly higher in humans given corn oil for two years than in controls receiving institution diet It has been suggested that the heart of a homeothermic animal stops beating because the fats solidify thus the heart should stop beating at a lower temperature if the animal has received a diet rich in unsaturated fats To test this hypothesis we cooled rats of which some had received a diet rich in saturated fats and others a diet rich in unsaturated fats

Methods and material

White rats of both sexes were used Five control animals were given hospital diet Twenty five animal were given a semisynthetic diet consisting of casein 125 g wheat starch 100 g corn oil 250 g salt mixture[1] 20 g and a vitamin mixture[2] Twenty five animals were given a diet with the same constituents but for the corn oil which wa.

[1] The salt mixtur contains in gm/kg diet $CaCO$ 1? 6? K_2HPO 7 58 Na_2HPO 6 14 $Ca(PO)_2$ 11 78 NaCl 7 41 $MgSO$ sicc 2 78 FeSO sicc 0 21 MnSO × 4 H O 0 3? KJ 0 03 $ZnCO_3$ 0 0? CuSO × ? H O 0 0?

[2] Six tablets each contain mg vitamin A 5 000 IE vitamin D 750 IE vitamin B_1 5 mg vitamin B 5 mg vitamin B 1 5 mg calcium pantothenate 8 mg PP factor 50 mg vitamin C 100 mg vitamin E ? mg

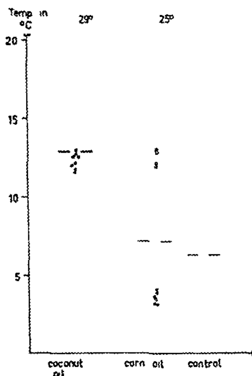


Fig 2 shows the temperatures for the last heart beat in rats receiving a semisynthetic diet containing 50 per cent saturated fats (coconut oil) and unsaturated fats (corn oil) and in control animals The mean in each series is indicated by — —

substituted with coconut oil One animal died in the first group of 25 and two in the second of 25 before the experiment was finished

Soon after the weaning period at an age of about 6—8 weeks the rats began the semisynthetic diet The animals were kept in cages with 5 rat in each In the corn oil series the time range for the experiments was March 8 till June 4 1960 for 15 animals and May 17 till July 20 1960 for 10 animals Corresponding values for the coconut oil series was April 20 till July 15 1960 for 10 animals and May 12 till July 15 1960 for 15 animals The body weight was measured at regular intervals (see Fig 1)

After varying times (see Fig 1) the animals were anesthetized with Nembutal in a dose adjusted to prevent shivering and cooled in a refrigeration box (BJORCK *et al* 1954) after they had been tracheotomized and a small glass cannula had been introduced into the trachea Artificial respiration with pure oxygen was begun at about 35 C and continued until term An oscilloscope (Elema) registered the electrocardiograms continuously and ECGs were recorded at an oesophageal temperature of 30 C 25 C 20 C 15 C and 10 C Usually leads I and II were recorded The body temperature was recorded by means of a thermocouple with the electrode situated in the oesophagus behind the heart (In earlier experiments we had determined the distance the electrode should be introduced to reach as close as possible the back surface of the heart) The oesophageal temperature was read when the last heart beat was recorded by the oscilloscope The oscilloscope screen was observed for a further five minutes so that there would be no doubt that it really was the last spontaneous electrical manifestation heart that had been observed Immediately after this the thorax was opened

Table I Iodine values for the different fractions in the three series

	Corn oil series	Coconut oil series	Control series
Cell nuclei and membranes	3 4	8.5	8 8
Mitochondria	10 5	9 4	15 0
Microsomes	7 9	23 0	—

heart touched with a forceps to see if this resulted in a contraction The heart was then removed and stored in absolute alcohol at −20 C for eventual fractioning into cell nuclei mitochondria and microsomes according to a method by SCHNEIDER (1956) then the iodine value of the different fractions was determined (HINSBERG 1936)

Results

The hearts from rats in the corn oil series beat spontaneously till a significantly lower temperature (mean 7 2° C) than the hearts in the coconut oil series (mean 12 9 C) The corresponding value for the controls was 6 3 C but there were only five animals in this group (fig 2) The difference between the corn oil and coconut oil series is highly significant (dof 45 t 3 7 $p < 0\ 001$) There was also a significant difference between the controls and the coconut oil series (dof 26 t 2 38 $0\ 025 > p > 0\ 02$)

The electrocardiograms showed no definite differences other than a tendency to a higher heart rate at corresponding temperatures in the corn oil series than in the coconut oil series No significant differences were found in the QRS-duration but the Q T-duration was slightly shorter at 15 C in the corn oil series than in the coconut oil series The T wave appearance and the number of arrhythmias were similar in the different series

Some of the hearts were pinched with forceps when they had been taken out Four of seven hearts in the coconut oil series beat on mechanical stimulation while seven of eight responded in the corn oil series

The iodine values for the three fractions in each series are given in Table I

Discussion

It is known that the surrounding temperature and the diet can change the iodine number and the melting point of the depot fats (FAWCETT and LYMAN 1954) Knowing that the turn over is high for the depot fats it is not surprising that also other tissues have a lipid composition dependant on diet (HORWITT HARVEY and CENTURY 1959) The present results show that the hearts from rats fed on diet containing a high amount of unsaturated fats (corn oil) beat at a significantly lower temperature than hearts from rats fed on diet with a high amount of saturated fats coconut oil) The exact mechanism for this differ

ence is unknown but remembering the results by HORWITT *et al* (1959) it is tempting to speculate that the lipids of the myocardial cell membranes in the corn oil series might have a lower melting point than the lipids in the coconut oil series

FAWCETT and LYMAN (1954) among others have shown that there is a correlation between the iodine value and the melting point and solidification range

The iodine values in Table I however show rather large variations and no significant difference between the groups In this connection it should be born in mind that iodine value determination is only approximate Gas chromatography as used by HORWITT *et al* (1959) gives a more detailed view

That different physical properties might have been of importance for the ability of the tissues to tolerate hypothermia is supported by the finding that it was more difficult to mechanically stimulate the hearts from the coconut oil series than those from the corn oil series after the last heart beat

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Control of Thyrotrophic Hormone (TSH) Secretion by the "Heat Loss Center"

By

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Abstract

ANDERSSON B L EKMAN C C GALE and J W SUNDSTEN *Control of thyrotrophic hormone (TSH) secretion by the Heat loss center* Acta physiol scand 1963 *59* 12—33 — In 9 goats studied local cooling of the preoptic/anterior hypothalamic region (the heat loss center) was found to cause a marked increase in the release of protein bound iodine (PBI[131]) from the thyroid gland concomitant with the development of extreme hyperthermia Local cooling of other parts of the brain was negative in these respects Median eminence lesions blocked the thyroidal response to central cooling The lesioning of the median eminence by RF heating however in itself resulted in a conspicuous release of PBI[131] from the thyroid Local warming of the heat loss center was found to block the thyroidal response to a general cold stress and to retard the normal release of PBI[131] from the thyroid When such warming was prolonged in the cold a lowering of the core temperature about 10 C occurred but the development of hypothermic hyperglycemia was prevented It is suggested that central warm detectors in the heat loss center even at a normal body temperature exert a certain inhibitory tone on the release of TSH from the hypophysis The strength of this inhibition seems to increase in proportion to the rise of the temperature of the heat loss center *i e* in proportion to the degree of activation of the central warm detectors It seems likely that other hormonal cold defense mechanisms are also inhibited in a similar manner by the heat loss center

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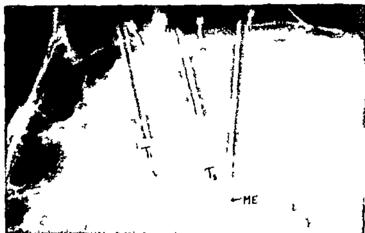


Fig 1 *An X ray picture of the head of Goat Tita taken from the side*

T = A silver thermode for cooling placed in the forebrain rostral to and in the caput of the right caudate nucleus (For details see Fig 3 A)
T = A similar thermode placed in the heat loss center i e medially in the preoptic/anterior hypothalamic region (For details see Fig 2)
ME = Two parallel needle applicators for temperature recording insulated but for 3 mm at the tips The tips of the applicators were riding over the median eminence They were used for RF coagulation of the median eminence in the nonanesthetized goat

Previously it was briefly reported that local cooling of the preoptic/anterior hypothalamic region of the goat causes conspicuous thyroid activation (ANDERSSON *et al* 1962 a and b) whereas local warming of the same part of the brain inhibits the thyroid activation normally occurring during general cold stress (ANDERSSON *et al* 1962 c) On the basis of these observations it was suggested that central warm detectors in the rostral hypothalamus (the 'heat loss center' of MAGOUN *et al* 1938) exert a tonic inhibition on the release of thyrotrophic hormone (TSH) from the adenohypophysis (ANDERSSON GALE and SUNDSTEN 1962 e) The importance of extra pituitary factors in the regulation of thyroid activity has however been demonstrated by SODERBERG (1958) Therefore it could not be accepted as certain that the thyroid activation observed during preoptic/anterior hypothalamic cooling was mediated via the pituitary

In the following a more detailed report of the studies of thyroid activity during central cooling and warming will be given Also the effect of median eminence lesions on thyroid activity and on thyroid response to central cooling will be described

Methods

Fourteen adult horned goats were used for the present study Eleven of the animals were used in experiments involving local brain cooling and three in experiments in which the preoptic/anterior hypothalamic region was locally warmed

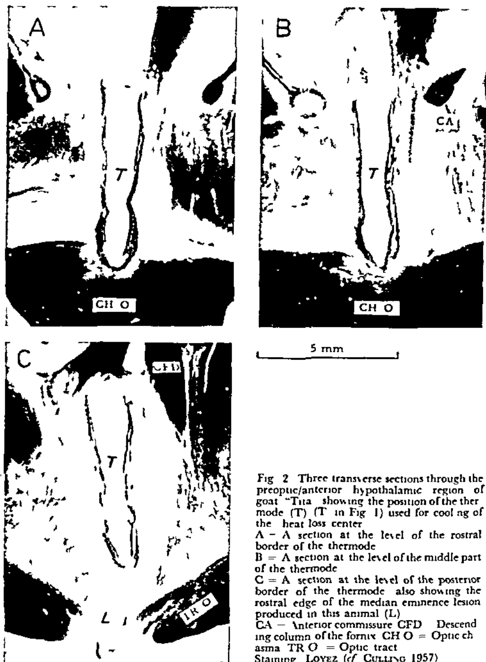


Fig 2 Three transverse sections through the preoptic/anterior hypothalamic region of goat "Tita" showing the position of the thermode (T) (T in Fig 1) used for cooling of the heat loss center
A – A section at the level of the rostral border of the thermode
B = A section at the level of the middle part of the thermode
C = A section at the level of the posterior border of the thermode also showing the rostral edge of the median eminence lesion produced in this animal (L)
CA – Anterior commissure CFD Descending column of the fornix CH O = Optic chiasma TR O = Optic tract
Staining LOYEZ (*cf* CULLING 1957)

Technique of local brain cooling Nine goats had a silver thermode for cooling implanted medially in the preoptic region and the anterior hypothalamus (Fig 1 T_2 and Fig 2) One of these goats had an additional thermode placed in the forebrain just rostral to and in the anterior part of the caput of the right caudate nucleus (Fig 1 T_1 and Fig

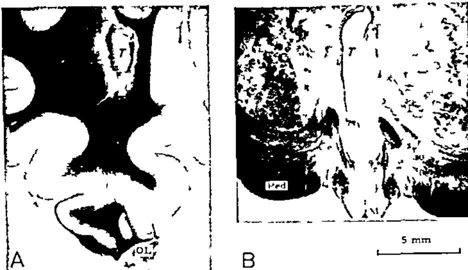


Fig 3 A A t ansverse section at the level of the rostral edge of the forebrain thermode (T) in goat Tita (T_1 in Fig 1)
OL = Olfactory tract
B A trans erse section through the mammillary bodies (M) showing the position of a thermode (T) placed med ally in the posterior thalamus and the rostral mesencephalon of one of the goats
Ped = Cerebral peduncle
Stain ng Loyez (cf Culling 1957)

3 A) Another goat had a thermode placed dorsal to the mammillary bodies medially in the posterior thalamus and the rostral mesencephalon (Fig 3 B) The construction of the thermodes and the methods of implantation and of central cooling were previ ously described (Andersson and Larsson 1961 Andersson Gale and Sundsten 1962 d) This technique allowed local brain cooling for long periods of time (a week or longer) in animals maintained with no additional restraint in their accustomed environment The degree of central cooling was controlled by use of a needle applicator for temperature recording permanently implanted with its thermosensitive tip at varying distances from the lateral surface of the thermodes An analysis of the data obtained in all centrally cooled animals showed that a perfusion of the entire cooling system at constant pressure and suction with water cooled to 1 C 1 2 m above the heads of the goats caused a drop of the temperature at the very surface of the thermode from 39 C to about 15 C 1 mm lateral to about 25 C 2 5 mm lateral to about 30 C and 5 mm lateral to about 35 C The steepest temperature gradient was thus found within 1 mm from the surface of the thermode On the basis of these very approximate calculations it may be concluded that in animals with the thermode placed medially just above the optic chiasma, the temperature of the entire preoptic region and of most of the anterior hypothalamus was lowered more than three degrees centigrade below the core temperature

Technique of local warming of the preoptic/anterior hypothalamic region

A special technique was developed to obtain as uniform warming as possible of the entire preoptic region and the anterior hypothalamus Two identical uninsulated circular silver plates were implanted under general anesthesia bilaterally into the in

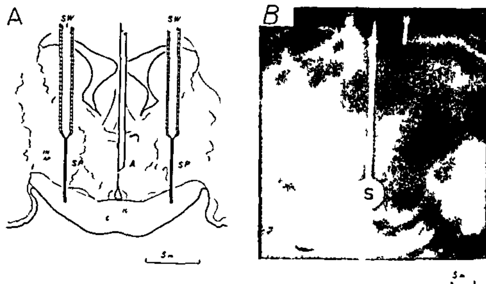


Fig 4 A drawing of a transverse section through the preoptic region (A) and an X ray picture (B) of the head of one of the goats to illustrate the arrangement for RF warming of the heat loss center
Sp and S = Two parallel uninsulated silver plates RF warming was applied between these plates On the X ray picture (taken from the side) the two plates are overlapping
A = The tip of a needle applicator for temperature recording placed in the center of the cylinder bounded by the silver plates
SW = Insulated silver wires connected with contacts on the horns of the animal
For further explanation see text

ternal capsula The diameter of the plates were 6 or 8 mm depending upon the size of the goat The silver plates were placed so deeply as to transect the dorsal part of the optic tracts on both sides The cylinder of brain tissue limited by the plates then included the entire preoptic region and the anterior part of the hypothalamus A needle applicator for temperature recording was also implanted with its thermosensitive tip in the center of the cylinder bounded by the silver plates The latter were attached to insulated silver wires connected with contacts on the horns of the goats Fig 4 illustrates the arrangement in a drawing of a transverse section through the preoptic region (A) and in an X ray picture of the head of one of the goats (B) A frequency of 27 Mc/s was used for warming the brain tissue between the plates The RF energy was generated by a converted 30 W crystal controlled U S Army surplus BC 604 transmitter All speech and modulation circuits were removed from the transmitter and a power supply was added The output power was regulated by varying the screen grid voltage of the final power amplifying stage The screen grid current was supplied by a cathode follower with the DC grid voltage set by a potentiometer The transmitter 30 Ω output impedance was matched by a quarter wave transformer to a 150 Ω twin lead feeder which carried the RF energy to a matching transformer on the head of the animal The matching transformer was found necessary because of the large capacitive component of the electrode impedance

Before the equipment was used for experiments in animals it was tested with the silver plates placed 0 7 to 1 cm apart in an egg white medium When the temperature in the center of the cylinder bounded by the plates was raised 10 C the rise in tem

perature within all other parts of the cylinder was found to be 8 to 10 C If the power output was further raised so that the temperature in the center of the cylinder became 70 C a coagulum was formed which filled the whole region between the silver plates but was not adherent to the latter The coagulation first started as an almost spherical body midway between the silver plates but very soon took the form of a cylinder It may therefore be assumed that the maximal rise in temperature during preoptic/anterior hypothalamic warming was equal to that actually recorded from the centrally implanted applicator In no experiment did this temperature rise above 42 5 C

One disadvantage with the technique was that the placement of the silver plates deep in the internal capsula interrupted fibres passing laterally from the preoptic region and the anterior hypothalamus and that it caused damage to the internal capsula and reduced the number of functioning visual fibres As a result all animals suffered from aphagia and adipsia and somewhat impaired vision during the first postoperative week The recovery was however almost complete three to four weeks after the operation

Technique of producing median eminence lesions in the nonanesthetized goat Two animals which had cooling thermodes implanted medially in the preoptic/anterior hypothalamic region, and which in previous experiments had been found to respond to central cooling with a conspicuous thyroid activation were chosen for median eminence lesions During general anesthesia two needle applicators for temperature recording (insulated but for 3 mm at the tip) were implanted bilaterally deep into the hypothalamus under X ray control so that the uninsulated tips were riding over the median eminence (Fig 1 ME) The needle applicators were fixed in the usual manner with dental cement onto the surface of the skull Several weeks later during an experimental period involving studies of thyroid activity the needle applicators were used to make median eminence lesions (see under results) The lesions were produced by applying RF energy between the uninsulated tips of the two needle applicators The temperature at the tips was in one animal maintained at 65 C for 4 min and in the other at 75 C for 7 min This heating of the median eminence was not seen to disturb or distress the animals An interruption of hypothalamo-hypophyseal connections however soon became apparent by the onset of diabetes insipidus which was most pronounced in the goat which had obtained the more intense heating

Studies of thyroid activity At the beginning of each experimental period involving studies of thyroid activity the goats were given 40 or 60 microcuries of I^{131} (as carrier free KI^{131}) by stomach tube In most experiments the thyroid radioactivity was measured at intervals by a scintillation counter The use of an extension tube on the crystal detector ensured that the measuring point was at the same distance (50 cm) from the thyroid gland each time a count was made Determinations of total plasma and plasma protein bound I^{131} (PBI^{131}) were continuously made during all the experiments Blood samples were drawn at intervals from the jugular vein into a heparinized syringe The blood was immediately centrifuged and the radioactivity of the plasma was determined in a well scintillation detector After the determination of total I^{131} in plasma the PBI^{131} was measured Protein precipitation was performed with 20 % trichloracetic acid The first periods of central cooling and of general cold stress (ruminal cooling) were generally not started until three or four days after the radioiodine had been given *i e* when the thyroid content had reached its maximum and had begun to fall Preoptic/anterior hypothalamic warming on the other hand in some instances was made within the first 48 hours after administration of radioiodine *i e* during the steepest rising phase of plasma PBI^{131} The animals were used for experiments involving studies of thyroid activity for up to 14 days after radioiodine had been given They were then allowed to rest for at least 9 additional days (usually much longer) before a new experimental period was started by the administration of radioiodine

Cold exposure and inducement of general hypothermia. In some instances the goats were exposed to cold by placing them in an environmental temperature of about 5° C. For the studies of thyroid activity it was however often found advantageous to induce a more rapid and intense cold stress. This was accomplished by giving the goats ice water (4 to 5 l, i.e. 0.12 l/kg b. w.) by stomach tube into the rumen.

Care and environment of the animals. The goats were routinely maintained collared in metabolism cages in an animal room and most experiments were performed in this their normal position and customary environment. During a period of three months in the autumn of 1962 the room temperature was kept as low as between 14 and 16° C, since a fairly low external temperature was found advantageous for the thyroid studies. Otherwise the temperature of the animal room was maintained between 16 and 20° C, generally as close to 18° C as possible. However, occasional temperature rises up to 23° C during short periods in the summer could not be avoided.

Histology. The goats sacrificed to date were decapitated under Nembutal anesthesia. Perfusion of the carotids was performed with physiological saline followed by 5 per cent formol saline. The brains were embedded in celloidin and cut in serial transverse sections 30 μ thick. Alternate sections were stained with toluidine blue and hematoxylin (LOYEZ cf. CULLING 1957). The pituitaries from the two goats with median eminence lesions were embedded in paraffin, sectioned serially 15 μ and stained according to LADEWIG's (1938) modification of MALLORY.

Blood sugar determinations. Blood sugar was determined according to the method of SOMOGYI (1945).

Results

I. Thyroid uptake of I^{131} and pre-experimental plasma PBI^{131} level

All goats used in the present series of experiments had a high thyroid uptake of radio-iodine. A maximum uptake of 40 to 90 per cent of the dose given was found on the third day after the administration of I^{131}. The pre-experimental PBI^{131} level in the blood plasma showed even greater variations from animal to animal ranging from 0.2 to 8 per cent of given dose per liter blood plasma on the third or fourth day after the administration of radio-iodine. The lowest plasma PBI^{131} values were generally found in the animals showing the highest radio-iodine uptake in the thyroid. This high uptake may perhaps be explained by the fact that all goats came from an iodine deficient part of Sweden (Harjedalen). Only occasionally was any extra supply of iodine given during the pre-experimental period.

II. Effects of preoptic anterior hypothalamic cooling

A) General thermoregulatory and alimentary responses. As observed in earlier experiments (ANDERSEN, ANDERSSON and GALE 1962) preoptic/anterior hypothalamic cooling as a rule caused shivering during the first hour when the experiment was performed in an external temperature below 18° C. Concomitant with the rise in core temperature this shivering became less and less intense and vanished completely when the rectal temperature of the goats had reached 40.5 to 41.5° C. Especially during the first three to four cooling periods in each animal shivering was also often observed at external temperatures

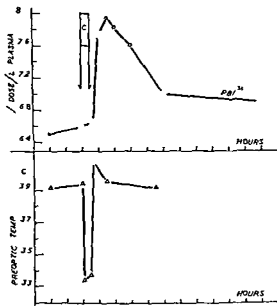


Fig 5 *Thyroidal response to a short period (30 min) of local cooling of the heat loss center*

Note that the release of PBI from the thyroid became apparent first after cessation of central cooling The preoptic temperature was recorded 4 mm lateral to the surface of the thermode

above 18 C but it was then usually weak and of short duration During preoptic/anterior hypothalamic cooling the core temperature of the goats within 1 1/2 to 2 hours rose from about 39 C to a level between 41 and 41 8 C It then remained at this high level during the entire period of central cooling

The alimentary response to preoptic/anterior hypothalamic cooling was similar to that obtained in previous studies (ANDERSSON and LARSSON 1961 ANDERSSON *et al* 1962 d) The animals thus refused to drink water during cooling periods not exceeding 36 hours but continued to eat hay to a normal extent in spite of a core temperature well above 41 C Two animals with the larger part of the thermode placed in the anterior hypothalamus showed a markedly increased appetite during central cooling but stopped eating within a minute or two after cessation of central cooling

B) Thyroid response to preoptic/anterior hypothalamic cooling All nine animals showed a pronounced thyroid activation during preoptic/anterior hypothalamic cooling Five of these goats having a fairly high pre experimental plasma PBI[131] level were selected for more detailed studies In these animals the first three hours of central cooling caused a rise in plasma PBI[131] by 55 to 125 per cent of the pre cooling value and a corresponding fall in thyroid radioactivity The rise in plasma PBI [1] became apparent after 30 min but the steepest rise generally occurred between 30 and 150 min after the onset of central cooling Therefore if the preoptic/anterior hypothalamic region was locally cooled for 30 min or less no rise in plasma PBI[131] was generally

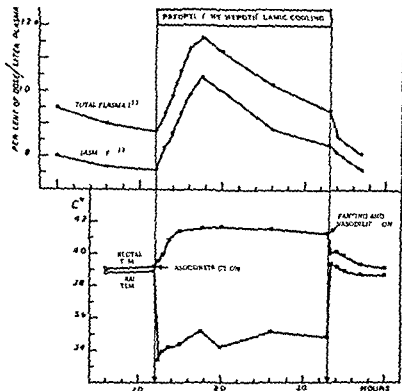


Fig 6 Thyroidal and other thermoregulatory effects of a prolonged period (?1 hours) of cooling of the heat loss center

Note that after (hours no further rise in plasma PBI[131] was obtained Room temperature 21 C Brain temperature recorded 4 mm lateral to the surface of the thermode

observed until after the actual cooling period (Fig 5) When relatively short periods of central cooling (2 to 3 hours) were repeated at 21 to 22 hours intervals a thyroid response of about the same magnitude was obtained each time During prolonged periods of preoptic/anterior hypothalamic cooling the plasma PBI[131] reached a maximum after 4 to 6 hours and then started to decline Although declining it remained well above the expected normal slope of the plasma PBI[131] curve during the entire period of central cooling When cooling was stopped the plasma PBI[131] returned to or fell below the expected normal value (Fig 6)

C) Effects of local cooling of other parts of the brain A comparison was made between the effects of equally intense cooling via each of the two thermodes of the goat Titt This animal had one thermode placed in the heat loss center, i e medially in the preoptic/anterior hypothalamic region (Fig 1 T_2 and Fig 2) and the other thermode placed in the forebrain just rostral to, and in the caput of the right caudate nucleus (Fig 1 T_1 and Fig 3 A)

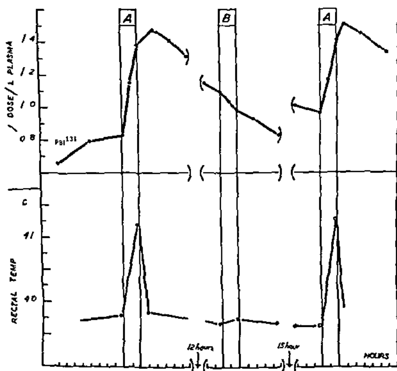


Fig 7 Thyroidal and core temperature responses to local cooling of the heat loss center for two hours (A) in comparison with the lack of effect of similar cooling in the forebrain (B) in the same animal

The result is shown in Fig 7 Local cooling of the heat loss center caused the expected thyroid activation and rise in general core temperature In contrast the forebrain cooling had none of these effects and furthermore failed to inhibit drinking

In another goat thyroid activity was studied during local cooling of the posterior thalamus and the anterior mesencephalon (Fig 3 B) Three hours of central cooling in this animal caused no significant change in plasma PBI131 but a general cold stress induced by ruminal cooling caused marked thyroid activation Local cooling of the posterior thalamus and anterior mesencephalon induced in this animal otherwise very lively a state of drowsiness Further within two minutes such cooling completely inhibited the strong shivering response elicited by intense ruminal cooling That this inhibition was reversible was shown by interrupting the central cooling for repeated short periods However huddling piloerection and peripheral vasoconstriction were not inhibited indicating that the central cooling in this animal blocked the motor shivering mechanism *per se*

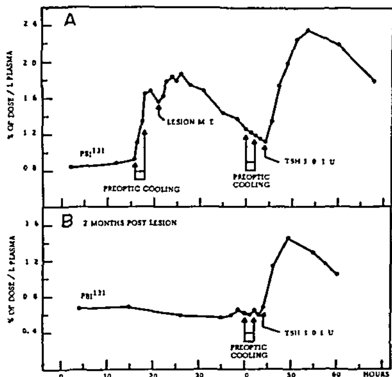


Fig 8 A The thyroidal response to local cooling of the heat loss center in goat "Tita (preoptic cooling) compared to the lack of response to similar cooling 20 hours after the median eminence was lesioned Note that the RF heating of the median eminence in itself resulted in a conspicuous thyroid activation
B The lack of response to cooling of the heat loss center in the same animal two months later Note that the subsequent intravenous injection of 1 IU of TSH caused conspicuous thyroid activation

III The effect of median eminence lesions on thyroid activity and on the thyroid response to local cooling of the heat loss center

The use of permanently implanted electrodes for RF lesioning of the median eminence made it possible to study the effect of such lesions in the unanesthetized goat during experimental periods involving studies of thyroid activity The two animals used in this study had in previous experiments shown a conspicuous thyroid activation during preoptic/anterior hypothalamic cooling

Three days after radioiodine had been given to the first goat ("Tita) central cooling was performed for three hours As expected the plasma PBI^{131} rose 100 per cent above the pre cooling value Two hours after cessation of central cooling when the plasma PBI^{131} had reached its maximum and had just started to fall the median eminence was lesioned by raising the temperature between the electrode tips to 65 C for 4 min This heating of the median eminence was not seen to disturb or distress the goat but it induced

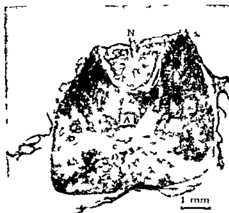


Fig 9 A section through the pituitary of goat Tita showing atrophy of the neurohypophysis (N) and central ischemic necrosis (ci) in the adenohypophysis (A) as effects of the median eminence lesion Staining LADEWIG (1937)

transient moderate polypnea apparently due to slight rise of the temperature also in the rostral hypothalamus The damage to the median eminence however within half an hour caused a second rise in the plasma PBI^{131} which continued for 8 hours and reached a somewhat higher level than that seen during the preceding period of central cooling The plasma PBI^{131} then gradually declined and almost reached the pre cooling level on the first post lesion day A renewed three hour period of central cooling then no longer had any effect on the thyroid activity but a subsequent intravenous injection of 3 I U TSH (Actyron Ferring) caused a conspicuous and longlasting thyroid activation The changes in plasma PBI^{131} during this experimental period in goat Tita are shown in Fig 8 A

Two months later the goat was again used for studies of thyroid activity The uptake of radioiodine in the thyroid and especially the plasma PBI^{131} level were then found to be abnormally low on the second day after administration of radio iodine probably due to impaired pituitary function caused by the median eminence lesion In order to speed up the uptake of radio iodine and to raise the plasma PBI^{131} level 0 5 I U of TSH was then given to the animal Four days later the preoptic/anterior hypothalamic region was cooled for three hours The central cooling caused a rise in body temperature but had no effect on the plasma PBI^{131} content A subsequent intravenous injection of 1 I U of TSH however caused a conspicuous and longlasting elevation of the PBI^{131} in the blood plasma (Fig 8 B)

Histological examination of the brain of goat Tita revealed a bilateral lesion in the median eminence (Fig 2 C shows the anterior edge of this lesion (L)) Examination of the pituitary showed almost complete atrophy of the neurohypophysis and central infarction in the adenohypophysis (Fig 9) The latter was probably the consequence of a damage to the median eminence

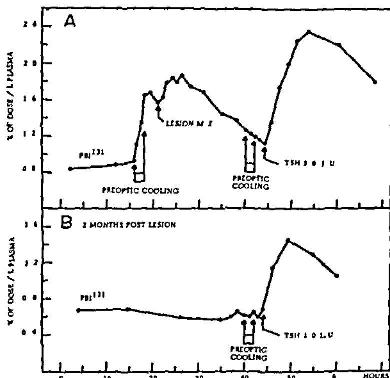


Fig 8 A The thyroidal response to local cooling of the heat loss cent r in goat "Tita (preoptic cooling) compared to the lack of response to similar cooling 70 hours after the median eminence was lesioned Note that the RF heating of the median eminence in itself resulted in a conspicuous thyroid activation

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plasma PBI[131] of 55 per cent above the pre lesion value (Fig 10 A) The plasma PBI[131] then remained at approximately the same level for 48 hours when a second rapid rise occurred increasing the plasma PBI[131] by another 30 per cent within three hours A three hour period of central cooling performed on the following day did not significantly change the plasma PBI[131] level but the subsequent intravenous injection of 0 5 I U of TSH caused conspicuous and longlasting thyroid activation (Fig 10 B) On the 10th post lesion day the "heat loss center" was again cooled for three hours This time also the central cooling had no effect on the plasma PBI[131] but again the injection of 1 I U of TSH caused a conspicuous thyroid activation

Histological examination of the brain and the pituitary of this goat revealed a large bilateral lesion in the median eminence central infarction in the adenohypophysis and total atrophy of the neurohypophysis The latter may account for the marked diabetes insipidus observed after the RF heating of the median eminence in this goat.

IV Preoptic/anterior hypothalamic warming

A) General thermoregulatory and metabolic effects All experiments involving RF warming of the preoptic/anterior hypothalamic region were primarily undertaken to study the thyroid response, but certain other thermoregulatory and metabolic effects were also studied. Special attention was thus payed to changes in general core temperature, peripheral circulation (ear surface temperature) respiratory rate shivering response during cold exposure and blood glucose level Therefore experiments involving central warming were at times also performed at external temperatures considerably above or below the usual 18 C

Like recent studies in the same (ANDERSSON PERSSON and STRÖM 1960) and other species (HAMMEL *et al* 1962 INGRAM and WHITTOW 1962) these experiments emphasized the inhibitory influence on the "heat loss center" exerted by the inflow from peripheral cold receptors. As also could be expected from earlier studies in cats (STRÖM 1950) and goats (ANDERSSON *et al* 1960) peripheral vasodilatation was found to be a much more sensitive index of activation of the "heat loss center" than was polypnea. However an even more sensitive index was the inhibition of shivering At a room temperature of about 18 C the preoptic/anterior hypothalamic temperature thus had to be raised 0 3 to 0.5 C to induce peripheral vasodilatation whereas a rise of 1 5 to 2 C was needed before a definite polypneic response was obtained (resp > 60 min) At a room temperature of 30 C, where ear vasodilatation was present in spite of a normal brain temperature marked polypnea was induced by raising the temperature between the implanted silver plates 0.5 to 1 C. During cold exposure (5 C) or during ruminal cooling the temperature of the "heat loss center" on the other hand had to be raised to or above 42 C before polypnea appeared Ear vasodilatation under these circumstances became apparent

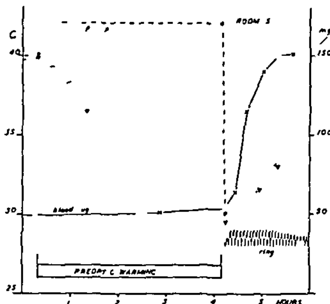


Fig 11 *Pronounced hypothermia elicited by local warming of the heat loss center in a cold (5° C) environment*

Polypnea and ear vasodilatation were present during the steep decline in rectal temperature Shivering and hypothermic hyperglycemia did not appear until after cessation of central warming

at about one degree centigrade lower preoptic/anterior hypothalamic temperature and shivering was completely inhibited at an even lower temperature

As also observed by HAMMEL *et al* (1962) the degree of alertness of the animals seemed to influence the sensitivity of the heat loss center In the drowsy goat a lesser rise of the temperature in the preoptic/anterior hypothalamic region was required to elicit panting than in the same animal during alertness

When the temperature of the preoptic/anterior hypothalamic region was maintained at 42 to 42 5 C for longer periods of time in a cold (5 C) environment or subsequent to the administration of cold water into the rumen first the polypnea and then the ear vasodilatation gradually disappeared Shivering however remained inhibited during the entire period of central warming even when this was prolonged for 6 hours As a result the body temperature of the goats dropped at first steeply and then more gradually to a level between 29 and 31 C At this low body temperature the animals were very drowsy and displayed irregular jerks of the head and the neck Although a core hypothermia of this severity is known to cause considerable blood sugar rise (FUHRMAN and CRISMON 1947) as long as preoptic/anterior hypothalamic warming was maintained the blood sugar remained at normal or very slightly elevated level On cessation of central warming shivering appeared after about three minutes

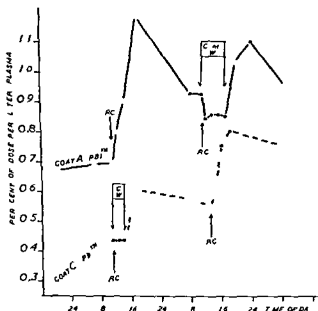


Fig 12 *Blockin of th thyroid response to ruminal coolin (RC) by local warming of th heat loss center (CW)*

In goat A simple ruminal cooling was performed on the fi st experimental day whereas ru minal cooling in combination with central warming was appli d on the second day In goat C the sequence was the reverse

and then gradually grew very strong At the same time the blood sugar started to rise and reached a level two to three times the normal within half to one hour (Fig 11)

B) Thyroidal effect In two of the goats (A and B) central warming was started 11 (A) and 36 (B) hours after the administration of radio iodine *i e* during the rising phase of plasma PBI^{131} In both animals preoptic/anterior hypothalamic warming caused a marked retardation of the plasma PBI^{131} rise followed by an accelerated rise on cessation of central warming The retardation of the plasma PBI^{131} rise was most pronounced in goat A (for details see ANDERSSON *et al* 1962 c)

Goat A and a third goat (C) were also used for studies of the influence of central warming on the thyroid response to a general cold stress On three consecutive days goat A was subjected to the same degree of ruminal cooling On the first and third day ruminal cooling was performed without warming the heat loss center This procedure caused a steep rise in plasma PBI^{131} during the following four hours On the second day ruminal cooling was per formed at the beginning of a six hour period of preoptic/anterior hypothalamic warming (to 41 5 C) This time the plasma PBI^{131} remained below the initial value during the entire six hour period of central warming but rose

steeply when the central warming was stopped (for details see ANDERSSON *et al* 1962 c) In goat C ruminal cooling was initially performed 5 minutes after commencement of a three hour period of preoptic/anterior hypothalamic warming (to 42 C) Although the experiment was performed during the rising phase of plasma PBI[131], the plasma PBI[131] remained unchanged during the three hours of central warming but started to rise again after cessation of central warming Renewed ruminal cooling was made in this animal the following day, this time without central warming Within three hours after the administration of cold water into the rumen, the plasma PBI[131] then rose by 45 per cent of the pre cooling value Fig 12 gives a comparison of the plasma PBI[131] changes in goat A and C during the first and second experimental days As can be seen in this figure the thyroid activation obtained by pure ruminal cooling was in both animals considerably greater than that observed after cessation of central warming in spite that the fall in general core temperature in the latter instance was much more pronounced A possible explanation may be that at the time central warming was stopped the initially ice cold water in the rumen had reached thermal equilibrium with the rest of the body Thus the inflow from ruminal cold receptors would have been considerable reduced at the time central warming was stopped

Discussion

In homoiotherms a relatively constant body temperature is maintained in spite of wide variations in the temperature of the external environment This obviously demands a complex interplay between neural and hormonal thermoregulatory factors The aim of most studies of temperature control however, has been to elucidate either the neural or the hormonal thermoregulatory mechanisms whereas relatively few experimental studies have helped to clarify how they act in conjunction with each other

Central warm detectors and peripheral cold receptors seem to play an essential role in the neural control of body temperature The classical experiments of MAGOUN *et al* (1938) focused interest on the preoptic/anterior hypothalamic region as a site for such central warm detectors The importance of this part of the brain as a heat loss center has later been confirmed and been further elucidated by numerous investigations culminating with the recent elegant microelectrode studies of NAKAYAMA and HARDY (1962) The latter experiments have completed and confirmed EULER s (1950) earlier electrophysiological studies which showed that the rostral hypothalamus does in fact contain elements which are specifically sensitive to a rise in body temperature The studies of NAKAYAMA and HARDY further show that at least some of these warm detectors are slightly active at brain temperatures considerably below normal and that their degree of activation increases concomitant with a rise in temperature much above normal level The present experiments and earlier studies (HEMINGWAY *et al* 1940 ANDERSSON GRANT

and LARSSON 1956 HAMMEL *et al* 1962) indicate that the central warm detectors not only serve to activate neural heat loss mechanisms but even more important they exert a brake on neural cold defense mechanisms especially shivering This may explain why in the goat an inactivation of the warm detectors by local cooling, elicits shivering only when the cold defense mechanisms are activated by an inflow from peripheral cold receptors or by certain other nervous or certain humoral factors (ANDERSEN *et al* 1962)

Another possible explanation for the shivering response to preoptic/anterior hypothalamic cooling would be that this part of the brain contains not only warm detectors but also hypothermia detectors responsible for the activation of the shivering mechanism and other cold defense reactions during hypothermia (HAMMEL, HARDY and FUSCO 1960) NAKAYAMA and HARDY (1962) however did not find elements within the heat loss center which increased their discharge by cooling According to HAMMEL *et al* (1962) certain neurons in the preoptic/anterior hypothalamic region may still be thought to act as hypothermia detectors in a more general physiological sense HAMMEL *et al* have thus suggested that there may be two different types of warm sensitive elements in this part of the brain one set of sensors having a low Q_{10} and an other having a high Q_{10} The low Q_{10} sensors (which would be discharging also at a brain temperature somewhat below normal) may serve to activate cold defense mechanisms whereas the high Q_{10} sensors (discharging mainly at a higher brain temperature) may have the opposite thermoregulatory task This theory is somewhat difficult to reconcile with the effect of RF destruction of the preoptic/anterior hypothalamic region in nonanesthetized goats Within a few minutes after such lesions have been produced strong and persistent shivering appears which rapidly causes a more than 2 C rise in the body temperature of the animals This shivering response is elicited in spite of the fact that all types of thermo detectors within the lesioned region of the brain have been destroyed (ANDERSSON and GALE 1963)

Like earlier studies in the same and other species (ANDERSSON *et al* 1960 HAMMEL *et al* 1962 INGRAM and WHITTOW 1962) the present experiments show that an increased inflow from peripheral cold receptors lowers the sensitivity of the heat loss center As mentioned above the shivering response to local cooling of this part of the brain is to a great extent also dependent upon the intensity of the inflow from peripheral cold receptors But other nervous factors as well seem to have an effect similar to that of the peripheral cold inflow Thus slight pain and emotional excitation may elicit periods of intense shivering during local cooling of the heat loss center (ANDERSEN *et al* 1962) Accordingly the temperature of the preoptic/anterior hypothalamic region must be raised to a higher level to induce peripheral vasodilatation and panting in the alerted than in the drowsy dog (HAMMEL *et al* 1962) This phenomenon was here also observed in the goat

The purely nervous factors involved in temperature regulation are thus

complex A similar complexity seems to apply to the purely hormonal factors The total picture of temperature control becomes even more complicated since it involves the interaction between nervous and hormonal mechanisms

The importance of hormonal factors in temperature regulation has been recognized for many years Half a century ago SEIDELL and FENGER (1912) observed that the environmental temperature influences thyroid function and as early as 1927 CANNON et al observed increased adrenaline secretion in animals exposed to cold On the basis of this observation CANNON et al suggested that the increase in extra muscular chemical heat production which occurs during cold exposure is due to the calorigenic effect of adrenaline Many experimental studies have later confirmed that cold exposure causes increased thyroid and adrenal medullary activation Apparently not only adrenaline but also noradrenaline is secreted in increased amounts under such circumstances (LEDUC 1961) The thyroid and the sympathetic nervous system, including the adrenal medulla thus appear to be important in the metabolic response to cold Although less certain, it is likely that increased adrenocortical activity is another link in this response EGDAHL and RICHARDS (1956) observed that acute exposure of dogs to severe cold causes a definite but rather shortlasting increase in adrenal corticoid secretion Further prolonged cold exposure has been found to cause adrenocortical hypertrophy (KATSH, KATSH and OSHER 1954)

Evidence has been produced that the different hormonal factors involved in the metabolic response to cold act in synergism with each other Thyroxin f ex seems to potentiate the calorigenic actions of adrenaline and noradrenaline which may be one important feature in cold adaptation (HSIEH and CARLSON 1957 HSIEH CARLSON and GRAY 1957) But hormonal factors apparently also potentiate purely nervous mechanisms involved in the cold defense Intravenous infusion of adrenal catecholamines was thus found to elicit shivering during local cooling of the heat loss center of goats maintained in a warm environment (ANDERSEN et al 1962)

In most studies of hormonal thermoregulatory mechanisms variations in the external temperature have been used as the experimental stimulus These changes in the external temperature have generally not much altered the core temperature of the animals The thyroid and adrenal activation observed during cold exposure has thus apparently been elicited mainly by the increased inflow from peripheral cold receptors Although this thyroid activation has been shown to be mediated via the hypothalamo pituitary axis (EULER and HOLMGREN 1956 KNIGGE and BIERMAN 1958) hitherto little has been learned to what extent variations in the temperature of the heat loss center may *per se* influence hormonal cold defense mechanisms The present experiments were therefore undertaken with the aim to study the importance of the heat loss center for the hormonal control of body temperature The results of these experiments justify the conclusion that thyroid activity is to a great

extent dependent upon variations in the temperature of this center From earlier experiments briefly reported (ANDERSSON *et al* 1962 a b c) no definite conclusions could be drawn whether this is an effect mediated via the anterior pituitary (TSH release) due to other humoural factors or dependent on a direct nervous influence on the thyroid The latter two possibilities could not be excluded in view of SODERBERG s (1958) finding that thyroid activity is directly affected by nervous and extra pituitary humoral factors However the effect on the thyroid of short periods of preoptic/anterior hypothalamic cooling (Fig 5) speaks in favour of this response being mediated via the pitui tary The time course of the response was thus indistinguishable from that observed after intravenous TSH injections An increase in thyroid activity was observed first 15 to 30 min after commencement of central cooling *i e* in some experiments not until central cooling had already been stopped More direct evidence that the adenohypophysis is involved in the response was provided by the effect of lesions in the median eminence Such lesions blocked the thyroid response to cooling of the heat loss center but they were not likely to have affected extra pituitary thyroid stimulating factors

Although the present series of experiments thus strongly suggests an in fluence of the preoptic/anterior hypothalamic temperature on the pituitary control of thyroid activity these experiments tell little of the importance of the peripheral cold receptors in the hormonal response to cold During cold exposure a certain thyroid activation apparently occurs in spite of a normal or at least initially even slightly elevated preoptic/anterior hypothalamic temperature Since all the present studies on thyroid activity were performed at external temperatures below 23 C, it is impossible to say whether or not a certain cold inflow from the periphery was needed to obtain the thyroid activation by central cooling Similar experiments performed in a thermally neutral or warm environment may better demonstrate the importance of the peripheral temperature in this respect

Since local cooling of the heat loss center caused a marked increase in thyroid activity in a constant external temperature it is reasonable to suggest that central warm detectors even at a normal brain temperature are suffi ciently active to exert a certain brake on the release of TSH from the adeno hypophysis NAKAYAMA and HARDY s (1962) studies showing that at least some of the thermosensitive elements in the preoptic/anterior hypothalamic region discharge at a normal and even subnormal brain temperature strengthen this suggestion The discharge rate of these sensors increases parallel to a rise in temperature much above normal level This is correlative to the present observation that local warming of the "heat loss center' increases the inhibi tion of the TSH release A rise of the temperature of the preoptic/anterior hypothalamic region above normal was thus found to retard the normal release of PBI^{131} from the thyroid and to block the thyroidal response to a general cold stress

complex A similar complexity seems to apply to the purely hormonal factors The total picture of temperature control becomes even more complicated since it involves the interaction between nervous and hormonal mechanisms

The importance of hormonal factors in temperature regulation has been recognized for many years Half a century ago SEIDELL and FENGER (1912) observed that the environmental temperature influences thyroid function and as early as 1927 CANNON *et al* observed increased adrenaline secretion in animals exposed to cold On the basis of this observation CANNON *et al* suggested that the increase in extra muscular chemical heat production which occurs during cold exposure is due to the calorigenic effect of adrenaline Many experimental studies have later confirmed that cold exposure causes increased thyroid and adrenal medullary activation Apparently not only adrenaline but also noradrenaline is secreted in increased amounts under such circumstances (LEDUC 1961) The thyroid and the sympathetic nervous system including the adrenal medulla thus appear to be important in the metabolic response to cold Although less certain it is likely that increased adrenocortical activity is another link in this response EGDAHL and RICHARDS (1956) observed that acute exposure of dogs to severe cold causes a definite but rather shortlasting increase in adrenal corticoid secretion Further prolonged cold exposure has been found to cause adrenocortical hypertrophy (KATSH KATSH and OSHER 1954)

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Effects of Bretylium, Reserpine, Guanethidine and Sympathetic Denervation on the Noradrenaline Content of the Rat Submaxillary Gland

By

M BENMILOUD[1] and U S v EULER

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Abstract

BENMILOUD M and U S VON EULER *Effects of bretylium reserpine guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland* Acta physiol scand 1963 *59* 34—42 — After sympathetic denervation the noradrenaline content of the rat submaxillary gland decreased slowly during the first 8 hours and disappeared completely in 24 hours Reserpine and guanethidine caused depletion of noradrenaline from denervated as well as innervated glands In the case of reserpine the denervated gland was slightly less depleted ($p < 0.05$) The noradrenaline depletion produced by treatment with these two drugs and by sympathetic denervation was significantly reduced by simultaneous injection of bretylium In addition bretylium had a releasing effect not as striking as that of reserpine and guanethidine but highly significant The implications of these results are discussed

Sympathetic denervation depletes the noradrenaline content of submaxillary glands (EULER and PURKHOLD 1951 STRÖMBLAD and NICKERSON 1961) as well as of other organs Likewise the disappearance of noradrenaline from different organs following treatment with reserpine (HOLZBAUER and VOGT 1956 CARLSSON and HILLARP 1956) and guanethidine (SHEPPARD and ZIMMERMAN 1959 SANAN and VOGT 1962) has been reported by several authors The action of bretylium is not as well documented though it has been shown that long term administration produces depletion (GREEN 1960) while acute treatment causes no change or an increase in the noradrenaline content of the heart

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Table I Noradrenaline content and weight of rat submaxillary glands intact and after sympathetic denervation. Significance of difference calculated on pairs

Denervation time hours	Average weight of rats g	Number of estimations (2—3 glands pooled)	Average weights of submax gland g			Average noradrenaline content µg/g gland		
			Controls	Denerv	Percent gain	Controls	Denerv	Percent remaining
0	277	2	0.28	0.29	2.7	1.56	1.48	94 ± 0.7
4	303	2	0.29	0.33	16	1.46	1.22	83 ± 3
8	293	3	0.28	0.37	33	1.49	1.10 *	75 ± 3.5
12	288	5	0.31	0.37	20	1.40	0.85 **	61 ± 6.8
16	308	5	0.30	0.32	8	1.42	0.51*	35 ± 5
24	290	2	0.31	0.33	7	1.32	0 *	0
48	318	3	0.26	0.30	14	1.28	0*	0
72	290	2	0.29	0.30	3	1.32	0**	0

p = 0.05* p = 0.01** p = 0.001***

(BRODIE and KUNTZMAN 1960) and other organs (RYD 1962). However an acute releasing effect on the heart noradrenaline has also been reported (GILLIS and NASH 1961).

In the present report the time course of noradrenaline depletion was studied after sympathetic denervation in the rat submaxillary glands. These paired organs were chosen because they provide a reliable control. In addition the effects of bretylium, reserpine and guanethidine were tested on the noradrenaline content of the gland 12 hours after denervation, at which time partial depletion had occurred. Although it is more or less accepted that reserpine acts peripherally, a central action has been described at least for the adrenal medulla (ERÄNKÖ and HOPSU 1958, STJÄRNE and SCHAPIRO 1958, HILLARP 1960, MIRKIN 1961) though not confirmed by others (CALLINGHAM and MANN 1958). Guanethidine acts peripherally since it does not enter the brain.

Finally we have investigated some aspects of the blocking action of bretylium on the noradrenaline depleting effect on guanethidine and reserpine, recently reported (NORTON and COLVILLE 1961, KUNTZMAN *et al.* 1962, RYD 1962).

Methods

Sprague Dawley male rats weighing from 270 to 350 g were used throughout this study.

Superior cervical ganglion extirpation. The rats were lightly anesthetized with ether. After a midline incision of the skin, the right side of the neck was bluntly dissected and

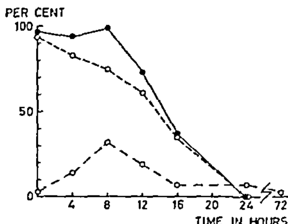


Fig 1 Effect of sympathetic denervation on the noradrenaline content of the submaxillary gland
A Per cent of noradrenaline left in the denervated gland corrected for weight increase
B Same as above uncorrected
C *Per cent of weight increase of the denervated gland*

the paratracheal muscles opened laterally to uncover the right carotid sheath The superior cervical ganglion was dissected free and entirely removed

Drugs All the drugs were given subcutaneously within 5 min after denervation Subsequent doses of bretylium were injected every 4 hours as indicated Bretylium 50 mg/kg Reserpine 20—30 μg/kg Guanethidine 20 mg/kg Bretylium tosylate was diluted in distilled water and reserpine in the commercial solvent Guanethidine and reserpine were kindly furnished by Ciba AB Vallingby Bretylium was a gift of the Wellcome laboratories

Extraction procedure After intervals varying from 0 to 72 hours the animals were killed by a blow on the neck, the submaxillary glands were removed and quickly weighed Ipsilateral glands of 2 rats were pooled in a few cases 3 rats were used Extraction was carried out after mincing in a total volume of 20 ml of 5 % trichloroacetic acid The catecholamines were adsorbed on aluminium hydroxide and eluted with 3 ml of 0 5 N acetic acid followed by an equal volume of 0 25 N acetic acid

Noradrenaline was estimated according to the method of EULER and LISHAJKO (1961) Only noradrenaline values are given here (as hydrochloride) since adrenaline was very low and the values therefore uncertain.

Results

Effect of denervation Table I and Fig 1 show the gradual decrease in nor adrenaline content in the submaxillary gland after denervation The per centage of remaining noradrenaline which is recorded in the last column in Table I was used to plot curve B in Fig 1 There is a definite increase of the depletion rate after 8 hours *The first part of the curve would even be hori* zontal (curve A Fig 1) if the increase in weight of the denervated gland due

Table II Effect of bretylium subcutaneously every 4 hours on intact and sympathetically denervated submaxillary glands 4 12 and 24 hours after denervation

Treatment (First dose at time of denervation)	Time after denervation	Average weights of rats g	Number of estimations (2—3 glands pooled)	Average weight of submax gland g		Average noradr content of submax gland, µg/g	
				Controls	Denerv	Controls	Denerv
—	4	303	2	0.29	0 33	1 46	1.22
Bretylium 1 × 50 mg/kg	4	330	3	0 31	0 31	1 45	1.36
—	12	288	5	0 31	0 37 *	1 40	0 85* *
Bretylium 3 × 50 mg/kg	*12*	*300*	*4*	*0 31*	*0.31*	*1 08*	*1 12*
—	24	290	2	0 31	0.33	1 32	0
Bretylium 6 × 50 mg/kg	24	340	3	0 32	0 31	0 74	0 49

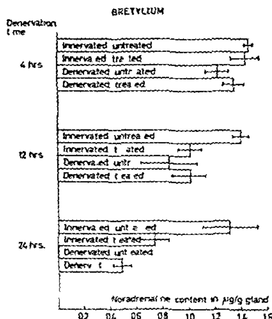


Fig 2 Effect of bretylium on the noradrenaline content (mean and S D) of innervated and denervated submaxillary glands Bretylium tosylate was injected subcutaneously (50 mg/kg) at the time of denervation. The same dose was repeated every 4 hours twice for the 12 hours group and 5 times for the 24 hours.

to swelling is taken into consideration This swelling reaches a maximum at 8 hours ($p < 0.01$) as can be seen in curve C Fig 1 At 24 hours the gland is completely depleted and this persists during the following 2 days

Effect of bretylium The results after bretylium treatment can be seen in Table II and Fig 2 After 4 hours there is no significant difference between untreated and bretylium treated animals Twelve hours after denervation bretylium has

Table III Effects of bretylium reserpine and guanethidine on innervated and sympathetically denervated submaxillary glands 12 hours after denervation

Treatment (at the time of denervation)	Average weight of rats g	Number of estimations (2—3 glands pooled)	Average weight of submax gland g		Average noradr content of submax gland µg/g	
			Controls	Denerv	Controls	Denerv
—	288	5	0 31	0 37 *	1 40	0 85 *
Bretylium 50 mg/kg	300	4	0 31	0 31	1 08	1 12
Guanethidine 20 mg/kg	309	4	0 32	0 33	0 13	0 11
Bretylium + guanethidine	316	4	0 29	0 29	0 85	0 71
Reserpine 20—30 µg/kg	310	8	0 34	0 38	0.31	0 43*
Reserpine + bretylium	297	5	0 30	0 30	0 87	0 90
Reserpine + guanethidine	330	2	0 32	0 37	0 08	0 05

caused a significant decrease in the noradrenaline content on the innervated side ($p < 0\ 001$) while the increase seen on the denervated side is not significant At 24 hours the difference between treated and untreated animals is still more marked in the innervated gland and has now become manifest also in the denervated gland

At 12 hours no difference was found between the effect of a single dose of bretylium 50 mg/kg and of 3 equally large doses given every 4 hours In one experiment a single dose of bretylium was less efficient after 24 hours than 5 × 50 mg/kg injected every 4 hours both in preventing the loss in the denervated gland and in depleting the innervated gland No swelling of the denervated gland occurred in the bretylium treated animals

Effect of reserpine Reserpine (Table III) causes depletion of the noradrenaline content on both sides However the residual amount is slightly higher on the denervated side ($p < 0\ 05$) and if the swelling not prevented by reserpine treatment were accounted for a higher significance would be found ($p < 0\ 02$) Simultaneous treatment with bretylium prevented significantly ($p < 0\ 001$) the reserpine effect on both sides although the values were lower than in the case of bretylium alone In a few experiments not reported reserpine was injected 4 hours prior to denervation with no remarkable difference in the result except for a somewhat more marked decrease

Effect of guanethidine Guanethidine (Table III) had a strong depleting effect on the noradrenaline content In this case no difference was observed between both sides Here again a concomitant injection of bretylium inhibited this effect, perhaps even more efficiently Combination of reserpine and guanethidine produced a similar fall as with guanethidine alone In the case of guanethidine as in the case of bretylium the swelling of the denervated gland was absent

Discussion

With regard to the time course of depletion after denervation our results are in agreement with those of WEINER, PERKINS and SIDMAN (1962) who reported a complete disappearance of noradrenaline from the rat brown fat tissues after 24 hours and no change at 8 hours KIRPEKAR CERVONI and FURCHGOTT (1962) however found no change at 24 hours and 95 % depletion at 36 hours in the cat nictitating membrane after superior cervical ganglion extirpation Whether this is a species or an organ difference cannot be stated as yet One can only speculate as to the exact mechanism of the noradrenaline disappearance after denervation but it seems probable that the repleting mechanisms are defective after the lapse of the latency period of 8 hours As we shall discuss later, the swelling of the gland paralleling the latency period is strongly in favour of continued sympathetic activity Apparently the stores at the nerve endings are still repleted during the first 8 hours Thereafter some change occurs possibly equivalent to degeneration After this latency period a decrease in transmitter stores is no longer compensated for It seems quite probable that the release is only a continuation of the normally occurring process This is supported by the fact that the disappearance rate is very similar to a zero order reaction resembling the finding of KUNTZMAN *et al* (1962) in the case of guanethidine

The depleting action of bretylium, if at variance with some reports (BRODIE and KUNTZMAN 1960 GREEN 1960 RYD 1962) substantiates the findings of GILLIS and NASH (1961) and others on the heart These discrepancies could be attributed to species or organ differences but we are inclined to think that they are also dependent upon the mode of treatment Most authors have used lower single doses of bretylium RYD (1962) used similar doses but intraperitoneally and not repeated At 4 hours we had no change in agreement with her findings in some organs At 24 hours the differences are highly significant this may be due to the spaced doses maintaining a fairly high level of bretylium in the nerves thus accentuating the releasing effect This is partially confirmed by CASS and SPRIGGS (1961) who found a qualitative change of the action of bretylium and guanethidine with time although they observed no decrease in the noradrenaline content after bretylium The doses used in the present study (50 mg/kg) were not unduly high since the general condition of the animals remained good

Even more interesting is the prevention by bretylium of the depletion normally occurring after sympathetic denervation This action substantiates the conclusion of BOURA and GREEN (1959) GREEN (1960) and EXLEY (1960) that bretylium blockade of sympathetic nerve action is exerted predominantly on the terminal portion of the neurons HERTTING AXELROD and PATRICK (1962) have shown that bretylium and guanethidine blocked the release of tritiated noradrenaline when sympathetic nerves were stimulated as well as after reser

pine treatment Thus it seems very likely that bretylium (like guanethidine) acts by blocking the normal release mechanism

The action of reserpine is interesting in several aspects Even very low doses of reserpine may cause a considerable depletion of the noradrenaline content of some organs (BRODIE and KUNTZMAN 1960) Our results that reserpine depletes noradrenaline after denervation of the organ are in agreement with the opinion that reserpine acts peripherally WEINER *et al* (1962) in a study on the rat brown fat bodies reported more noradrenaline on the denervated side 2 1/2 hours after reserpine while after 3 1/2 hours both sides were emptied The antagonistic action of bretylium on the reserpine depletion confirms the findings of NORTON and COLVILLE (1961) and RYD (1962) with brain liver heart, and spleen of the guinea pig It is in disagreement with KUNTZMAN *et al* (1962) who claim there was no such an effect However no data as to organs were given

With regard to guanethidine denervation did not influence its depleting action while bretylium strongly reduced it A similar effect was noted in the rat heart by KUNTZMAN *et al* (1962) It will be noted that the depletion was more marked with guanethidine than with reserpine in the doses used thus equal doses of bretylium seem slightly more efficient in counteracting the effect of guanethidine The combination of reserpine and guanethidine was slightly more active than the most efficient of the two drugs The interval after treatment was evidently too long to allow the early bretylium like effect of guanethidine reported by BURN (1961) and CASS and SPRIGGS (1961) to be seen Some preliminary experiments on rats pretreated with guanethidine one hour before sacrifice indicate the existence of a partial block of the reserpine induced disappearance of noradrenaline

Incidentally these experiments have put into light a swelling of the submaxillary gland which has been studied in more details by WELLS HANDELMAN and MILGRAM (1961) They claim that it is due to tissue hypertrophy since dry and wet weight were similarly increased and there was also some histological evidence of hypertrophy In addition they have found that denervation bretylium guanethidine and reserpine prevented the swelling occurring after incisor amputation This event could be mimicked by isoproterenol infusion (WELLS 1962) and in this case it was not prevented by denervation It seems probable that the increase of weight is secondary to release of noradrenaline in the gland consequently depletion of the stores would prevent it It is noteworthy that in our experiments in contrast to reserpine bretylium and guanethidine prevent the swelling induced by denervation We are tempted to relate this action to the blockade of the release of nerve transmitter caused by these two drugs

From the results of these experiments it appears that both bretylium and guanethidine have a reserpine like depleting effect on the noradrenaline in the rat submaxillary gland All three drugs act peripherally although reserpine

also exerts a central effect In the present study bretylium stands apart by its striking inhibition of the noradrenaline release due to reserpine guanethidine and sympathetic denervation Although not conclusive this speaks in favor of a similar mechanism of release under these three conditions in accelerating the liberation of amines at the nerve endings and/or preventing the repletion Possibly in the case of reserpine the rate of depletion would be modified by its secondary central action and the direct releasing effect of high doses on adrenergic nerve granules demonstrated in vitro by EULER and LISHAJKO (1960) On the other hand the blocking of noradrenaline release from granules in vitro with low concentrations of reserpine (EULER and LISHAJKO 1961) might indicate disturbed resynthesis as a main cause of depletion Guanethidine is differentiated further from reserpine by its bretylium like effect reported by BURN (1961) CASS and SPRIGGS (1961) and HERTTING *et al* (1962) and seen by ourselves in some preliminary experiments We propose this alternative to the theory of KUNTZMAN *et al* (1962) who advocate a complete difference in the mechanism of action of these drugs One of their main arguments was the lack of counteraction by bretylium of reserpine effect but this has been observed by us and others Both bretylium and guanethidine have a somewhat similar dual action but where the former appears to be mostly a blocking agent the depleting action of the latter is predominant

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The Effect of Prostaglandin from Human Seminal Fluid on the Motility of the Non-Pregnant Human Uterus in Vitro

By

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Received 22 December 1962

Abstract

Bygdeman M and R Eliasson *The effect of prostaglandin from human seminal fluid on the motility of the non pregnant human uterus in vitro* Acta physiol scand 1963 *59* 43—51 — Immediately following surgical removal of the uterus four myometrial strips of equal size were cut out from the corpus and used simultaneously each suspended in a 40 ml cuvette Temperature pH etc were continuously controlled The motility was recorded isometrically on a smoked drum Histological determination of the phase in the menstrual cycle was made on each uterus Prostaglandin from pooled human semen (HSF PG) inhibited the myometrium in most cases in a concentration of 0 005 U/ml bath fluid the highest sensitivity occurring at the time of ovulation Some uterus strips reacted by stimulation to prostaglandin Among these some (mid-cycle preparations) were inhibited by larger doses of prostaglandin while the remainder equally distributed with in the menstrual cycle were consistently stimulated Factors regulating the reactivity pattern are briefly discussed.

The pharmacodynamic properties of the human semen were described by Kurzrok and Lieb (1930) by Goldblatt (1933 1935) and by Euler (1934) Euler (1936 1939) demonstrated that the active principle by its biological and chemical properties could be differentiated from all other known smooth muscle stimulating autopharmacological compounds and it was provisionally named "prostaglandin So far it has only been demonstrated in semen and extracts from some of the genital organs of man sheep and goat (Euler 1934

1939 EULER and HAMMARSTRÖM 1937, ELIASSON 1959) In man and sheep it is secreted only from the seminal vesicles and the vesicular glands respectively (ELIASSON 1959) It is further the only smooth muscle stimulating factor of any importance in human emen (ELIASSON 1959, 1961)

EULER (1936 1939) demonstrated that the active factor in sheep vesicular gland extract was a nitrogen free unsaturated organic acid More recently BERGSTRÖM and his co-workers have confirmed this and isolated several smooth muscle stimulating compounds from the vesicular glands of the sheep and also determined their chemical structure (BERGSTRÖM and SJÖVALL 1960 a b BERGSTRÖM *et al* 1962) These have been called prostaglandin E F etc (PGE PGF, etc) Some difference between the action of total extracts and isolated factors have been noted however (BERGSTROM *et al* 1959)

Since it now has been demonstrated that prostaglandin most likely consists of several active compounds the following abbreviation will be used in order to differentiate total extracts from the various crystalline compounds A total prostaglandin extract from human seminal fluid will be named HSF PG the corresponding extract from sheep vesicular gland SVG PG If not otherwise is stated the partial purification has been performed according to ELIASSON (1959)

The biological activity of HSF PG is given in units (EULER 1939) At bio-assay on rabbit blood pressure and on the isolated rabbit jejunum one unit corresponds to about 10 μg of the crystalline compound PGE (BERGSTROM *et al* 1959)

The effect of human semen on the motility of the isolated human myometrium was first investigated by KURZROK and LIEB (1930) and COCKRILL, KURZROK and MILLER (1935) They could demonstrate that the normal response was that of inhibition On the other hand some uterine preparations consistently responded with an increased tonus when seminal fluid was added to the bath Moreover the authors found a close correlation between the last mentioned type of reaction and infertility

More recently ELIASSON (1959) has shown that HSF PG has the same effect on the isolated myometrium as the seminal fluid He could also demonstrate that some uterine preparations which responded with increased activity to a small amount of HSF PG changed their reactivity pattern to inhibition when a slightly larger dose HSF PG was added to the bath

In this paper the results of experiments with various doses of HSF PG on strips taken from uteri under different hormonal influences will be presented

Method

The myometrium has been obtained from patients undergoing hysterectomy due to myomatous changes in the uterus Immediately following the surgical removal of the uterus suitable pieces of apparently normal myometrium were taken longitudinally from the corpus uteri and suspended in cold Ringer s solution Four strips of equal size

Table I Number of strips and uteri in the different groups in the present study

Hormonal phase	Group					
	1	2	3	4	5	6
	Early middle proliferative phase	Late proliferative phase	Early secretory phase	Middle-late secretory phase	Metropathia hemorr cystica	Menopause
N of uteri	17	14	12	8	5	4
N of strips	50	30	32	19	1?	14

(about 20 × 2 × 2 mm) were then cut out each mounted in separate 40 ml cuvettes in an isolated organ bath This could be done within one hour In all cases part of the uteri were sent to histological examination for determination of the phase in menstrual cycle and the diagnosis

The salt solution used was Tyrode modified according to GENELL (1937) and aerated with 5 per cent CO_2 in O_2 The gas flow was adjusted to give a pH of 7 35 ± 0 05 controlled continuously or at 15 min intervals. The bath fluid passed continuously from below at a constant rate of 1 5 ml/min The bath temperature was kept at 37 5 C by a temperature controlled water bath The motility of the four strips was recorded almost isometrically using frontal writing levers with an amplification of 25 1 on a smoked drum

After 2—3 hours the spontaneous motility usually reached a steady state and the effect of HSF PG could be studied The test solution was diluted 10 times in Tyrode solutions taken from the cuvette immediately before it was added to the same cuvette in a volume of 1 0 ml The amount of HSF PG administered usually did not exceed 0 05 units per ml bath fluid in the cuvette

The HSF PG preparation used was extracted from pooled human seminal fluid (250 —500 ml) with 3 volumes of acidified acetone (1 ml 3 N HCl per 100 ml) The extract was filtered and evaporated until all acetone has disappeared The pH was adjusted to 7 5 with NaOH The activity was bio-assayed on isolated rabbit jejunum and expressed in units (EULER 1939 ELIASSON 1959) The total extract was divided in small portions corresponding to the amount required in one experiment and kept at — 20 C

The sensitivity of the myometrium was estimated in all experiments and expressed as HSF PG ID_{50} units per ml *i e* the lowest amount tested that caused a decrease in the amplitude of the contractions with 50 per cent or more during at least 10 min This was accomplished by adding increasing amounts of HSF PG starting with amounts that were below the probable threshold Moreover in those cases where the highest amount of prostaglandin tested (usually about 0 05—0 1 unit per ml) did not cause an inhibition this dose has been taken as ID in the statistical evaluation. This procedure is acceptable when the rank sum test by DIXON and MASSEY (1957) is used as in this investigation

Results

The 157 uteri strips investigated in this study were prepared from 60 uteri They were divided in groups according to the phase in the menstrual cycle determined by the histological examination Group 1 corresponds to early middle proliferative phase (approx 1—10 day) group 2 to late proliferative

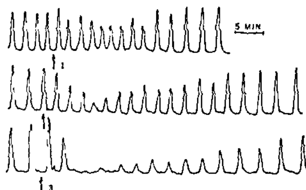


Fig 1 The dose response of the isolated human myometrium to HSF PG The myometrium is in late proliferative phase
1 = 0 003 U/ml
2 = 0 01 U/ml
3 = 0 03 U/ml

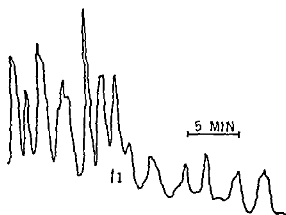


Fig 2 The effect of HSF PG on the motility of the isolated human myometrium in early secretory phase
1 = 0 0075 U/ml

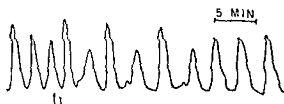


Fig 3 The lack of effect of HSF PG on the motility of the isolated human myometrium in late secretory phase
1 0 033 U/ml

phase (approx 10—14 day) group 3 to early secretory phase (approx 14—20 day) and group 4 to middle and late secretory phase (approx 20—28 day) Strips from uteri belonging to patients in menopause or with hormonal disturbances as metropathia hemorrhagica cystica (MHC) were placed in two special groups 5 and 6 respectively (Table I)

An inhibition of the spontaneous motility following prostaglandin was obtained in 90 per cent of the strips The dose prostaglandin needed for an obvious effect varied however in the different groups The myometrium is most sensitive around the ovulation time *i e* group 2 and 3 Usually 0 003

Table II Variation in sensitivity of the non pregnant human myometrium in vitro to prostaglandin in relation to the menstrual cycle

Group	No of uteri	Phase in menstrual cycle	Prostaglandin ID$_{50}$ (units/ml)	
			Median values	Range
1	17	Early middle proliferatory	0.015	0.0025 — 0.05
2	14	Late proliferatory	0.0045	0.001 — 0.01
3	12	Early secretory	0.007	0.0025 — 0.03
4	8	Middle late secretory	0.072	0.01 — 0.15

Table III Statistical significance in the difference of sensitivity between groups of uterine strips from various periods of the menstrual cycle to prostaglandin The group numbers correspond to the different phases of the menstrual cycle according to Table II

Comparison between groups	Degree of significance (P value)
1 and 2	0.01
2 and 3	No diff
2 and 4	< 0.001
1 and 4	No diff

Table IV Variation in sensitivity to prostaglandin of human myometrium in relation to time after the onset of the menopause

Years after the last normal menstruation	Number of experiments		Mean value of prostaglandin ID$_{50}$ (units/ml)
	Uteri	Strips	
1	1	4	0.0095
2	1	4	0.005
4	1	2	0.03
5	1	4	0.075

unit per ml bath fluid is sufficient to produce an effect (Fig 1 and 2) Fig 1 also illustrates the good dose response relationship During the other phases of the menstrual cycle the responsiveness to HSF PG is much less and some preparations did not respond even to the highest doses used (Fig 3) The sensitivity of the myometrium is 3—5 times higher at ovulation time than early and late in the menstrual cycle

The variation in sensitivity of the myometrium to HSF PG under various conditions is illustrated in Table II III and IV The figures in Table II are calculated from the median values for each uteri which are based on records from 2 to 4 strips From the statistical evaluation (Table III) it is apparent that

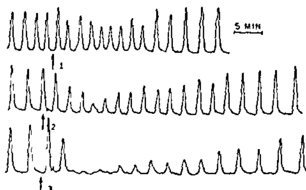


Fig 1 The dose response of the isolated human myometrium to HSF PG The myometrium is in late proliferative phase
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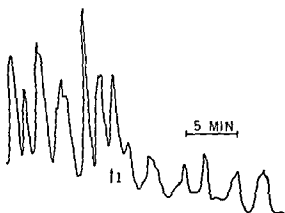


Fig 2 The effect of HSF PG on the motility of the isolated human myometrium in early secretory phase
1 = 0 0075 U/ml

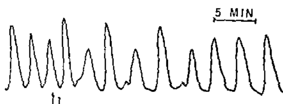


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An inhibition of the spontaneous motility following prostaglandin was obtained in 90 per cent of the strips The dose prostaglandin needed for an obvious effect varied however in the different groups The myometrium is most sensitive around the ovulation time *i e* group 2 and 3 Usually 0 005

from 7 uteri, all of which had been obtained from patients in mid-cycle The second type of reactivity pattern is illustrated in Fig 5 Both a moderate and a high dose HSF PG caused an increase in tonus and amplitude of the contrac tions This reactivity pattern was seen in 16 strips from 6 uteri equally distributed between groups 1 3 and 4

Discussion

In the present investigation it has been shown that the motility of the isolated human myometrium from women in fertile age can be affected by 0 001—0 005 units of HSF PG per ml bath fluid On the isolated uterus the activity of one unit HSF PG is approximately equivalent to 10 μg of the crystalline compound PGE_1 isolated by BERGSTROM and SJOVALL (1960) from the vesicular glands of sheep (BYGDEMAN and ELIASSON to be published) The sensitivity of the human myometrium to HSF PG is therefore at mid cycle very high Assuming that the chief active principle in HSF PG is PGE_1 0 01—0 05 μg per ml bath fluid cause a definite effect Corresponding figures for some other uterine active autopharmacological compounds are vasopressin 0 001—0 003 μg per ml (BYGDEMAN & ELIASSON to be published) acetylcholine 0 5—1 0 μg per ml and adrenaline 0 5—1 0 μg per ml (SANDBERG *et al* 1958)

In contrast to what has been reported for other autopharmacological substances the sensitivity of the myometrium varies to HSF PG during the menstrual period The difference in sensitivity between strips from mid cycle and those from early and late phases of the cycle is statistically highly significant On the other hand in the investigations concerning the effect of vasopressin acetyl choline and other substances on the motility of the isolated human myometrium a similar difference in activity with regard to phases in the menstrual cycle is not known A corresponding change in sensitivity of the myometrium to other sub stances can however not be excluded

KURZROK and LIEB (1930) and COCKRILL MILLER and KURZROK (1935) demonstrated that human seminal fluid usually caused an inhibition of the spontaneous motility of the isolated human myometrium They could also demonstrate that some uterine preparations responded with stimulation to some specimens of human semen and with inhibition to other specimens Finally there was a small group of uteri that regularly responded with increase in tonus to all semen samples tested

A stimulatory effect of HSF PG was observed in our study in some preparations from all 4 groups in the menstrual cycle At mid cycle the stimulatory effect was only obtained with very small doses and a slight increase in the amount always caused inhibition of the motility On the other hand certain preparations from the other phases of the cycle responded with stimulation to both medium and high doses of HSF PG It therefore appears that the re activity pattern of the myometrium is dependent both on the sensitivity of the muscle cells and the dose used Our results are in good agreement with those

obtained by KURZROK and LIEB (1930) since they used separate semen samples for their experiments and as it is known that the amount of prostaglandin in semen samples from different men is highly variable (ASPLUND 1947, ELIASSON 1959).

ELIASSON and POSSE (1961) studied the effect of HSF-PG on the uterine motility *in vivo* and found that when instilled intravaginally at the estimated time of ovulation in fertile women it usually caused a stimulation of the motility. In some cases the increased motility was followed by a marked inhibition. This phenomenon was thought to be a result of a postulated continuous absorption of HSF-PG from the vagina gradually increasing the blood concentration. The reactivity pattern would then be comparable with that observed *in vitro*, i. e. a small dose of HSF-PG stimulating and a slightly larger dose inhibiting the motility of the same preparation. At other phases of the menstrual cycle the uterus was much less sensitive to HSF-PG and the effect irregular.

The reactivity pattern of the human myometrium to HSF-PG *in vivo* is apparently similar to that *in vitro*. More thorough analysis on the mode of action of HSF-PG on isolated uterine strips therefore seems justified.

The fact that the myometrium is most sensitive to HSF-PG at ovulation time indicates that this factor plays a role in human reproduction. Further studies on the physiological function is therefore highly warranted.

We would like to express our gratitude to Dr. Å. LINDGREN for the histological examinations, to Dr. B. LINDSTRÖM for valuable advice in the statistical evaluation, to Miss A. WALLERINK for her skillful technical assistance, to the personnel at the Allmänna Barnbördshuset for collection of uterine preparations, and to Miss B. JONSSON for collecting the semen samples. Financial support from the Lalor Foundation, the Karolinska Institutet, and the Lars Hiertas Minne is thankfully acknowledged.

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Acta physiol. scand 1963 59 52—61

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Studies on the Elimination of Exogenous Lipids from the Blood Stream The Kinetics of the Elimination of a Fat Emulsion and of Chylomicrones in the Dog after Single Injection

By

Lars A Carlson and *Dag Hallberg*

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Abstract

Carlson L A and D Hallberg *Studies on the elimination of exogenous lipids from the blood stream* Acta physiol scand 1963 59 52—61 — The kinetics of the elimination from the blood stream of intravenously injected fat emulsion (Intralipid®) and of chylomicrones have been studied in the fasting dog The elimination was estimated by determination of the arterial whole blood glyceride concentration and subtraction of the basal glyceride concentration The same type of elimination was found in all dogs studied The elimination consisted of two phases operating above and below a glyceride concentration of around 1 1 mM (increase above basal level) respectively Above the concentration 1 1 mM the elimination was linear i e a constant amount of glycerides was eliminated per unit time The mean value for the elimination in this phase was 0 05 mmole/l blood per minute Below this concentration the elimination was single exponential i e a constant fraction was removed per unit time The mean value found for this elimination was 5 per cent per minute No greater difference was observed between the synthetic emulsion and chylomicrones The physiological significance of the maximal elimination rate above 1 1 mM and the concentration dependent elimination below is briefly discussed

The glyceride concentration in the blood during alimentary lipemia and during intravenous administration of fat emulsions is regulated by the amount of fat entering and leaving the blood stream per unit time At present we do not know in detail the mechanism controlling the rate of elimination of exog

enous lipids from the blood stream nor do we know if chylomicrones and different fat emulsions are handled principally in the same way A rapid removal of the chylomicrones from the blood stream in the rat was indicated from the work with labelled fatty acids by BERGSTRÖM BORGSTRÖM and ROTTENBERG (1952) Several authors have in subsequent experiments studied the removal of labelled chylomicrones injected i v (HAVEL and GOLDFIEN 1956 FRENCH and MORRIS 1957 BORGSTRÖM and JORDAN 1959) In general curves have been obtained which have been interpreted to indicate a rate of removal directly proportional to the concentration Calculated half life times have varied with the amount of administered chylomicrone glyceride generally with a range between 5—25 min However it has been pointed out that the disappearance curves obtained after chylomicrone injection are complex probably consisting of different phases (FRENCH MORRIS and ROBINSON 1956 FREDRICKSON MCCOLLESTER and ONO 1958)

The disappearance of i v administered fat emulsion has been studied by different authors WADELL et al (1953 a b) found that the plasma turbidity decreased in an exponential fashion after the i v administration of several different types of fat emulsions injected at a dose of 2 5 g/kg body weight in the rat The half lifes were 30 min or more MCCANDLESS and ZILVERSMIT (1958) injected labelled fat emulsion in smaller amounts in the dog and calculated the half life to be around 1—5 min EDGREN (1960) found an exponential decrease of the turbidity in dog blood after administration of more than 1 g o fat/kg body weight EDGREN furthermore found that the removal rate constant varied with the dose

During preliminary studies on the removal of fat emulsions from the blood in man we found that the elimination curves were complex and varied considerably from case to case These findings initiated the present study in the fasting dog where we have studied the arterial blood concentration of glycerides during the elimination of a fat emulsion and of chylomicrones It was found that the kinetics for the removal of this emulsion could be described by two simple equations in a manner not previously described for the elimination of exogenous fat

Materials and methods

Animals Young adult healthy mongrel dogs weighing between 10—14 kg were used Two *types of experiments* A and *B* were performed In *A* three dogs were studied once They were anesthetized with Nembutal 25 mg/kg body weight intratracheal intubation was performed the abdomen opened and splenectomy done Thereafter catheters were inserted into the portal vein through the splenic vein into the liver vein through the femoral vein and into the femoral artery The fat emulsion was injected through the artery at a dose of 0 1 g of glycerides/kg body weight within 30 sec Thereafter repeated samples were drawn from the different catheters the total amount of blood withdrawn amounting to 50—100 ml Two of the dogs were fasted for 36 hours and one fasted over night The data from the portal vein and the liver vein will not be included here

Table I. Rate constants for the removal of injected fat from the blood stream in fasted dogs

Dog no.	Dose given (g/kg)	K_1[1] (mmoles glycerides/l blood/min)	K_2[2] (%/min)	$C = \frac{K_1 \cdot 100}{K_2}$ (mmoles glycerides/l blood)
	Intralipid			
1	0.40	0.070	5.0	1.40
	0.30	0.061	5.3	1.15
	0.30	0.057	5.0	1.14
	0.20	0.067	[illegible]	1.22
	0.10	—	6.3	—
2	0.60	0.061	5.0	1.22
	0.30	0.060	5.0	1.20
	0.20	0.050	4.6	1.09
3	0.60	0.042	—	—
	0.30	0.046	4.1	1.12
	0.20	0.053	4.3	1.23
4	0.60	0.076	—	—
	0.10	—	8.7	—
5	0.60	0.034	3.3	1.03
6	0.50	0.048	4.3	1.12
7	0.35	0.033	3.2	1.03
	0.[illegible]	0.034	3.2	1.19
8	0.[illegible]	0.037	3.3	1.12
9	0.60	0.028	—	—
	0.0[illegible]	—	6.5	—
10	0.[illegible]	0.059	5.1	1.16
Mean		0.051	4.9	1.10
	Chylomicrones			
1	0.[illegible]	0.067	8.1	0.83
7	0.3	0.055	6.3	0.87
10	0.[illegible]	0.072	6.3	1.30
Mean		0.063	6.9	1.00

[1] K_1 = Maximal removal rate, i.e. removal rate for the linear phase.

[2] K_2 = Fractional removal rate, i.e. removal rate for the exponential phase.

C = "Critical concentration", i.e. the glyceride concentration above the basal concentration at which the elimination changes from linear to exponential.

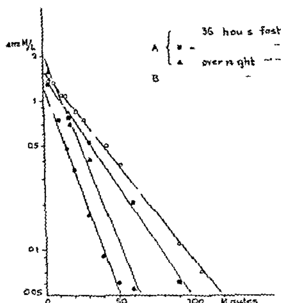


Fig 1 Elimination from the blood stream of single injection of 0 1 g glycerides/kg of a fat emulsion (Intralipid©) in dogs fasted 36 hrs or over night The glyceride concentration is given for whole blood and represents the increase above the basal level

In *B* the dogs were anesthetized and intubated as described above and a catheter inserted into the femoral artery The emulsion was injected in varying amounts (Table I) through this catheter during 30—90 sec and the catheter rinsed by flushing with saline Arterial blood samples about 3 ml were collected into heparinized tubes In this series 10 dogs were used Each dog was used in 1—6 expts (Table I) The interval between the experiments varied from 3—7 days The dogs were fasted over night and the experiment performed in the morning

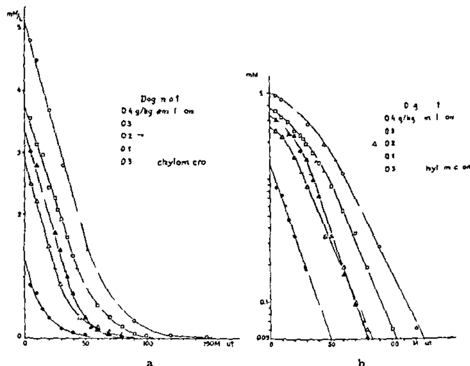
In both A and B two blood samples with 5—10 min interval was taken before the injection

Fat emulsion The emulsion used was Intralipid©[1] which has the following composition 10 per cent soya bean oil 1 2 per cent lecithin and 2 5 per cent glycerol (SCHUBERTH and WRETLIND 1961) The same batch of emulsion was used for all experiments

Chylomicrones Chylomicrones were obtained by cannulation of the thoracic duct in a donor dog The dog was given 400 ml of Intralipid© containing 20 per cent soya bean oil by mouth before the anesthesia The lymph was collected into iced bottles allowed to clot and centrifuged for 30 min at 15 000 *g* The infranatant was discarded and the fatty layer aspirated and reemulsified into saline The chylomicrones thus prepared were used within 12 hours

Lipid analysis The blood samples were extracted in duplicate immediately after withdrawal by pipetting 0 5 or 1 ml whole blood into tubes and thereafter 5 ml of methanol added with stirring The glycerides were then determined in principle according to CARLSON and WADSTROM 1959 and CARLSON 1959 in a slightly simplified way (CARLSON unpublished) After precipitation with methanol exactly 10 ml of chloroform is added while stirring Then 15 ml of saline is added and the tubes allowed to stand over night During this period all methanol diffuses into the upper phase An aliquot of the chloro-

[1] The emulsion was kindly supplied by A B Vitrum Stockholm



a b

Fig 2 a Elimination from the blood stream of various amounts of single injected fat emulsions and chylomicrones in dog 1 B The glyceride concentration is given as in Fig 1 The intercept on the curves represent the critical concentration i e where the elimination changes from linear to exponential (see text)

b The same data as in Fig 2 a but plotted on a semilogarithmic scale

form phase is aspirated with a syringe (6—8 ml) approximately 300—500 mg of activated silicic acid added and the tubes are centrifuged for some minutes at slow speed This procedure gives a complete extraction and removes all the phospholipids Thereafter aliquots are taken from the chloroform phase for the determination of glyceride glycerol (Carlson 1959)

Statistical analysis These were made as recommended by Snedecor (1957)

Results

The removal of 0 1 g of fat/kg body weight

The results of single injection of 0 1 g of fat/kg in the dogs in experiment A and in one dog in experiment B is given in Fig 1

The glyceride concentration is expressed as increase above the pre injection level The removal of the injected glycerides followed a single exponential curve in all cases during the time studied The removal rate was faster in the non operated dogs The data have been subjected to linear regression analysis which showed that the linear regression coefficient (b) is highly significant in all cases (all t values above 17)

The removal of different amounts of fat

In these experiments comparable results were obtained in all dogs studied. The results from dog 1 B are given in Fig. 2 a and b where the increase in blood glycerides above the pre-injection level is plotted against time on a linear and on a logarithmic scale respectively. It can be seen that the dose 0.1 g/kg was removed along a single exponential course. At a higher dose the removal was complex. Inspection of the linear and semilogarithmic plots reveals that the removal curve above a blood glyceride concentration of about 1.1—1.4 mM above the fasting level has a linear course while the curve below this concentration has an exponential course. Statistical analysis by means of linear regression analysis has shown these different lines to be highly significant. Fig. 2 b also reveals that the exponential elimination phase had the same slope for all doses given. Furthermore it can be seen that chylomicrones were eliminated in principally the same way as the fat emulsion.

The values for the slopes of these two different lines obtained in 24 expts. in 10 dogs are presented in Table I, where K_1 is the slope for the linear elimination phase expressed as mmoles/liter blood/minute and K_2 the slope for the exponential phase in the semilogarithmic plot expressed as fractional decrease/minute. It is seen that K_1 as well as K_2 is independent of the dose administered. Furthermore, any greater differences between the synthetic emulsion and the chylomicrones are not apparent.

Discussion

One difficulty in the study of elimination of injected glycerides from the blood stream is that the chemical method used here for the determination does not allow a differentiation between glycerides of different origin. Thus the determination will include unchanged injected glycerides, endogenous glycerides and exogenous glycerides taken up by various tissues and recirculated, if present. The same difficulty is present to some extent if one uses labelled glycerides and follows the concentration of label. In the present study we have chosen to subtract the initial fasting glyceride level from the values found after the injection of the emulsion in order to get the amount of exogenous glycerides present in the blood. It is recognized that this procedure involves the assumption that the sum of the endogenous and eventually re-circulated exogenous glycerides is constant during the experiment. It has not been possible to prove or disprove that this was the fact under our experimental conditions. However, the facts that the glyceride level returned rapidly to the basal level and the last part of the elimination curves had a strictly linear course either in linear or in semilogarithmic plot appear to justify this assumption for practical purpose. Furthermore, when HAVEL and FREDRICKSON injected labelled chylomicrones into dogs, they found the bulk of the radioactivity in the glycerides in the isolated chylomicrones and only small amounts

of activity in the isolated lipoproteins (HAVEL and FREDRICKSON 1956) This finding suggests that any recirculation of the chylomicrones in the form o lipoprotein bound glycerides would not effect the 'basal' glyceride level to any greater extent

After injection of fat emulsions at a dose of 0 1 g/kg body weight the elimination curves were all single exponential The operated animals on which splenectomy had been performed, all had a slower rate of elimination than the non operated dogs If this is due to the trauma *per se* or to the splenectomy or to both is not known *It has been shown that splenectomy decreases the re* moval rate of fat emulsions in the rat (WADELL et al 1953 a b)

After injection of fat emulsion at a dose of 0 2 g/kg or more the elimination curve could be resolved into two phases The first of these was linear and the second appearing below a concentration of about 1mM was single exponential During the first phase i e at a concentration above 1mM the amount of glycerides leaving the blood stream was thus constant per unit time and during the latter it was directly proportional to the concentration *It thus* appears likely that above 1 mM a maximal removing capacity was operating The mean value for this maximal capacity was found to be 0 05 mmoles/l blood/minute In a given dog this maximal capacity was independent of the administered dose If we assume a blood volume of 100 ml/kg body weight and a mean molecular weight of the glycerides of 900 this maximal capacity would correspond to 7 g/kg/24 hours It has recently been shown that dogs tolerate the administration of 6 g/kg/day of Intralipid© for 28 days without developing lipemia However if the daily administration is increased to 9 g/kg one of 4 dogs became lipemic (EDGREN et al 1962) indicating that the daily maximal fat tolerance for this emulsion in the dog is between 6 and 9 g/kg/day *The data obtained from the long term studies are thus in* close agreement with the maximal elimination capacity we have found here after single injection of the emulsion The correspondence between these two studies suggests that the single injection technique might be useful for evalua tion of the daily tolerance for a fat emulsion

The same principal type of elimination was found for chylomicrones The elimination rate for the two different phases was of the same order of magnitude for Intralipid and for chylomicrones Due to the limited number of tests it has not been possible to evaluate the possibility of minor differences in rates of elimination between Intralipid© and chylomicrones

The linear type of elimination found here has not been described previously for i v injected lipid Representative data from the literature have been collected and tabulated in Table II It is seen that in almost all cases the type of elimination is described as exponential except by FREDRICKSON McCOLLESTER and ONO who described the curve as complex There is no linear type of elimination described The difference between our results and others can

Table II Data from the literature concerning the elimination of intravenously administered lipids

Authors	Species	Nutritional state	Material	Dose (g/kg)	Method of analysis	Time interval studied	Type of elimination according to authors	Elimination rate ($T_{1/2}$)
French and Morris (1957)	Rat	Fasting	Chylomicron C'	0.05–0.04	Isotope	180 min	Exponential	Inversely proportional to the injected amount
Wadell et al (1953 a b)	Rat	Fed	Various emul.	2.5	Turbidity	270 min	First order reaction	Emulsion dependent
Borgstrom and Jordan (1959)	Rat	CH Fed	Chylomicron	0.1	Isotope	40 min	Exponential	13 min
Borgstrom George and Olivecrona (1961)	Rat	CH Fed	Chylomicron	—	Isotope	30 min	Exponential	5.3–9 min
Fredrickson McCollester and Ono (1958)	Dog	CH Fed	Chylomicron C⁴	0.013–0.08	Separation and isotope	60 min	Complex, initial phase exponential	2.5–4.5 min
Havel and Fredrickson (1956)	Dog	Fasting	Chylomicron C	0.33–0.6	Separation and isotope	40 min	First order reaction	11–24 min
Edgren (1960)	Dog	Non fasting	Emul.	1–7.5	Turbidity	30 hrs	Single exponential regression	Inversely proportional to the amount
Shafrir Sussman and Steinberg (1959)	Dog	Fed	Chylomicron C	0.1	Separation and isotope	30 min	Exponential	6.4–8.6 min
Havel and Goldfien (1961)	Dog	Fasting	Chylomicron C'	0.03	Isotope	60 min	Exponential	15–26 min
Goldman and Chaikoff (1950)	Dog	—	Emul	0.12	Isotope	8 hrs	Exponential	40–43 min
Kauste (1958)	Children	Fasting	Emul.	0.5	Total lipids	4 hrs	Exponential	30 min

CH = carbohydrate

probably be explained not only by the use of different fat emulsions as we see this type of elimination also with chylomicrones

It should be pointed out, however that the only authors who have given 0 2 g of fat/kg or more to dogs are HAVEL and FREDRICKSON (1956) and EDGREN (1960) HAVEL and FREDRICKSON followed the elimination of the chylomicrones only for 40 min and that time might not be long enough to permit a more definite statement about the nature of the curve EDGREN s experiments differ from ours in two respects Firstly another type of emulsion was given and second ly the dogs were not in the fasting state

In Table I is the concentration of the blood glycerides called C where the curve changed from the linear type to the exponential type

This concentration is defined as that concentration at which the fractional elimination per minute equals the maximal elimination rate per minute according to the formula

$$\frac{k_2 \cdot C}{100} = k_1$$

This concentration was found to be similar for the fat emulsion used here and the chylomicrones and is around 1 mmole/l

The physiological background to the observed types of elimination might be briefly commented upon The exponential phase is a common type of elim ination in biological systems and needs no further comment The linear phase on the other hand has not been described very often A linear type of elimination from the blood stream has been described for galactose (DOMIN GUERTZ and POMERENE 1944) and for ethanol (GOLDBERG 1943)

The linear elimination of ethanol was interpreted as maximal removal rate ited by the amount of available liver enzyme alcoholdehydrogenase (GOLD-BERG 1962)

The distribution of injected chylomicrones was studied by BRAGDON and GORDON (1958) There are several sites for the primary uptake of exogenous lipids from the blood stream They found that in fasting rats the liver took up a great part of the injected material However when the nutritional state was changed from fasting to carbohydrate fed they found that adipose tissue also took up a great part of the injected material It should be pointed out here that our dogs were fasted and that we have observed that the elimination curves are much more complex in carbohydrate fed dogs (unpublished results) Interestingly BELFRAGE BORGSTROM and OLIVECRONA (1963) have recently found in rats that the maximal liver uptake of injected glycerides is direcly proportional to the amount injected up to a certain limit (around 0 15 g/kg) above which limit the maximal uptake is not increased This might have a bearing to our finding of a maximal elimination rate above a dose of 0 1 g/kg for the dog

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Effect of Sympathetic Denervation and Adrenalectomy on the Catecholamine Content of the Rat Submaxillary Gland

By

U. S. v. EULER and G. RYD

Received 16 January 1963

Abstract

EULER, U. S. v. and G. RYD. *Effect of sympathetic denervation and adrenalectomy on the catecholamine content of the rat submaxillary gland.* Acta physiol. scand. 1963. *59.* 62—66. — In confirmation of earlier reports, cervical ganglionectomy causes a strong decrease in the noradrenaline content of the submaxillary gland while the adrenaline content is well maintained. In contrast to a recent report in the literature, the adrenaline content of the denervated gland was not found to be markedly influenced by adrenalectomy.

After postsynaptic sympathetic denervation the catecholamine content of mammalian organs falls to very low values (CANNON and LISSÁK 1939, GOODALL 1951). EULER and PURKHOLD (1951) and GOODALL and KIRSHNER (1956) also noticed that while noradrenaline almost disappeared from the organ after denervation the adrenaline content, although low, was well maintained, suggesting that the two amines are stored at different sites. This finding has been confirmed by STRÖMBLAD (1960). In a more recent paper STRÖMBLAD and NICKERSON (1961) have reported that the adrenaline content of the submaxillary gland of the rat disappears if the adrenals have been extirpated in addition to postsynaptic denervation, which was taken to indicate that the adrenaline content in the denervated organ depends on uptake of adrenaline from the adrenals. According to EULER (1951) the adrenaline occurring in mammalian organs is due to the presence of chromaffin cells. Whether innervated or not

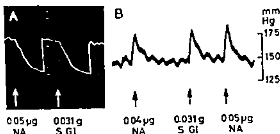


Fig 1 A Isolated chicken rectal caecum 5 ml bath 0 05 μg noradrenaline Extract of 0 031 g submaxillary gland from adrenalectomized rats B Blood pressure cat 0 04 μg noradrenaline 0 031 g submaxillary gland 0 05 μg noradrenaline.

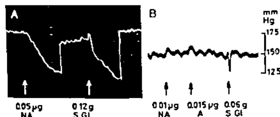


Fig 2 A Isolated chicken rectal caecum 5 ml bath 0 05 μg noradrenaline (NA) Extract of 0 12 g submaxillary gland from ganglionectomized and adrenalectomized rats B Blood pressure cat 0 01 μg noradrenaline 0 015 μg adrenaline 0 06 g submaxillary gland

these cells would be expected to maintain their catecholamine content after section and degeneration of sympathetic fibres running to the organ The adrenaline content of the organ should in such a case only be influenced to a small degree if at all by adrenalectomy In order to test the validity of this assumption we have repeated the experiments of STRÖMBLAD and NICKERSON using a different analytical technique

Methods

Sprague Dawley rats weighing about 350—400 g were used The superior cervical ganglion was extirpated on the left side under light nembutal anesthesia Bilateral adrenalectomy was performed by the dorsal approach and the animals maintained on 1 NaCl in the drinking water Six days after the operations the animals were sacrificed and the submaxillary glands from 6 or 7 rats removed weighed and extracted with 5 per cent trichloroacetic acid after thorough mincing

After 15 min extraction the extracts were centrifuged and filtered and passed through an aluminium oxide column after adjustment to pH 8 3 The catecholamines were eluted from the column with 3 + 3 ml 0 25 N H SO The eluates were adjusted to pH 5 with sodium hydrogen carbonate and the catecholamines estimated biologically on the blood pressure of the cat in nembutal anesthesia and on the isolated chicken rectal caecum The cat was pretreated with atropine 2 mg/kg ergotamine tartrate 0 1 mg/kg anthisan 5 mg/kg and cocaine hydrochloride 8 mg/kg in order to improve sensitivity (KARKI 1956) The eluates were tested against noradrenaline hydrochloride as standard and the activity ratios for noradrenaline and adrenaline determined The catecholamine amounts were computed according to EULER (1949)

Catecholamine estimations were made on the submaxillary glands of the following groups

Acta physiol scand 1963 59 62—66

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Effect of Sympathetic Denervation and Adrenalectomy on the Catecholamine Content of the Rat Submaxillary Gland

By

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Abstract

Euler U S v and G Ryd *Effect of sympathetic denervation and adrenalectomy on the catecholamine content of the rat submaxillary gland* Acta physiol scand 1963 59 62—66 — In confirmation of earlier reports cervical ganglionectomy causes a strong decrease in the nor adrenaline content of the submaxillary gland while the adrenaline content is well maintained In contrast to a recent report in the literature the adrenaline content of the denervated gland was not found to be markedly influenced by adrenalectomy

After postsynaptic sympathetic denervation the catecholamine content of mammalian organs falls to very low values (Cannon and Lissák 1939 Goodall 1951) Euler and Purkhold (1951) and Goodall and Kirshner (1956) also noticed that while noradrenaline almost disappeared from the organ after denervation the adrenaline content although low, was well maintained suggesting that the two amines are stored at different sites This finding has been confirmed by Strömblad (1960) In a more recent paper Strömblad and Nickerson (1961) have reported that the adrenaline content of the submaxillary gland of the rat disappears if the adrenals have been extirpated in addition to postsynaptic denervation which was taken to indicate that the adrenaline content in the denervated organ depends on uptake of adrenaline from the adrenals According to Euler (1951) the adrenaline occurring in mammalian organs is due to the presence of chromaffin cells Whether innervated or not

Discussion

The earlier finding that adrenaline remains in an organ after degeneration of its adrenergic nerves has been confirmed by STRÖMBLAD (1960) and by STRÖMBLAD and NICKERSON (1961) These authors consider as a possible explanation of this finding that the adrenaline remaining in the organ is due to uptake from circulating amines and storage at extraneural sites This hypothesis received support by their experimental results from which it emerged that adrenaline disappeared after removal of the chief adrenaline supply the adrenal glands

The discrepancy between their results and ours may be due to methodological differences It should be noted that the adrenaline content is small less than 0 1 µg/g From a methodological point of view estimation of small amounts of adrenaline in the presence of noradrenaline is a difficult problem involving some analytical hazards STRÖMBLAD and NICKERSON used a fluorimetric technique and estimated the catecholamines by the trihydroxyindole method In our experience this technique tends to give relatively large variations in the values of that amine in a mixture which occurs in a small proportion unless the readings are fairly high Under such circumstances the high sensitivity and specificity of a suitable pair of biological test preparations is of great value particularly in the estimation of small amounts of adrenaline in the presence of noradrenaline The cat blood pressure and the chicken rectal caecum have proven especially useful, and the relative proportions found with this technique in various organs have agreed well with the results obtained by the trihydroxyindole fluorimetric method when this has been used under favourable conditions

It might be argued that if adrenaline is taken up in a denervated organ from circulating adrenaline (deriving from the adrenal medulla) circulating noradrenaline should be taken up in a similar way especially since the noradrenaline concentration in plasma is normally considerably higher than that of adrenaline (VENDSALU 1960) From the results of STRÖMBLAD and NICKERSON (1961) it emerges however that the noradrenaline content of the gland after denervation is negligibly small or absent

The noradrenaline values reported in the present paper are open to some doubt since the biological effect of the eluates on the cat s blood pressure is atypical The values are therefore to be regarded as nominal all which can be said is that they are very low If we accept the noradrenaline values of STRÖMBLAD and NICKERSON (1961) as probably more correct it follows however that the adrenaline values in our series will be still a little higher

The present results therefore support our contention that the adrenaline present in organs after adrenergic denervation is due to an inherent adrenaline store and not dependent on an uptake from circulating adrenaline originating from the adrenals This concept is also indirectly supported by the finding of STRÖMBLAD and NICKERSON (1961) that no such uptake has been demonstrated

for noradrenaline This conclusion does not contradict the possibility that very small amounts of both amines may be taken up at extraneuronal sites from circulating amines under the prevailing conditions

From Table I it may be seen that the average noradrenaline values are higher in the adrenalectomized groups than in the controls This difference may be partly accounted for by the lower weight of the gland secondary to the adrenalectomy (cf WELLS HANDELMAN and MILGRAM 1961 BENMILOUD and EULER 1963) Higher noradrenaline values after demedullation in the submaxillary gland can also be found in the paper by STRÖMBLAD and NICKERSON (1961)

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Suppression by Thyroxine of the Thyroidal Response to Local Cooling of the "Heat Loss Center"

By

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Abstract

ANDERSSON B C C GALE and A OHGA *Suppression by thyroxine of the thyroidal response to local cooling of the heat loss center* Acta physiol scand 1963 *59* 67—73 — The intravenous injection of thyroxine (about 15 μg/kg b wt) was found to have blocked completely the thyroidal response to local cooling of the heat loss center after 2 hours in the goat However 15 to 20 min after the administration of thyroxine this blockage was still largely incomplete suggesting that an accumulation of thyroxine has to occur at some site before a full inhibition of TSH release is obtained The results are discussed in the light of BROWN GRANT s (1957) feed back hypothesis of the control of thyroid function

Much of our knowledge of nervous system control of endocrine activity has derived from the fundamental studies of HARRIS and coworkers (cf HARRIS 1948 1955) showing that nervous stimuli influence the secretion of pituitary trophic hormones via the hypothalamus and a humoral link in the hypothalamo hypophyseal portal vessels Such a mechanism is apparently responsible for thyroid activation during cold exposure (EULER and HOLMGREN 1956 b) Using electrical stimulation HARRIS and WOODS (1958) have found that the

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region of the hypothalamus which apparently directly controls thyrotrophic hormone (TSH) secretion is the anterior part of the median eminence and tuber cinereum But they find it inadvisable to consider this part of the hypothalamus a "center" regulating TSH secretion since it most likely represents a neural mechanism akin to a final common path by which other parts of the central nervous system may influence the secretion of TSH HARRIS and WOODS mention e g that the adjacent preoptic/anterior hypothalamic heat loss center (MAGOUN *et al* 1938) may influence the secretion of TSH via this "final common path Direct experimental evidence that such is really the case has recently been provided by the observation that local cooling of the "heat loss center causes conspicuous thyroid activation in the unanesthetized goat (ANDERSSON *et al* 1962 a and b) whereas local warming of this "center has the opposite thyroidal effect (ANDERSSON *et al* 1962 c) The thyroidal response to local cooling of the "heat loss center is apparently due to an increased release of TSH since it is blocked by lesions in the median eminence (ANDERSSON *et al* 1963)

It has been known for long time that there exists a reciprocal relationship between the secretion of TSH and thyroxine (cf PITT RIVERS and TATA 1959) An increased secretion of thyroid hormone or the administration of exogenous thyroxine thus lead to decreased secretion of TSH from the adenohypophysis This is apparently mainly due to a direct feed back action of thyroidal hormone upon anterior pituitary tissue (EULER and HOLMGREN 1956 a, BROWN GRANT HARRIS and REICHLIN 1957) although a certain action of thyroxine also on the hypothalamus can not be excluded HARRIS and WOODS (1958) have thus found that the excitatory effect of hypothalamic stimulation apparently predominates over the negative feed back of a raised blood level of thyroid hormone It therefore seemed to be of particular interest to study whether administration of thyroxine would effect the thyroid response to local cooling of the heat loss center

Methods

Two femal goats (A and B body weight 36 and 32 kg respectively) were used for this study The animals had silver thermodes for local brain cooling permanently implanted medially in the preoptic/anterior hypothalamic region. The methods of implantation and of central cooling were previously described (ANDERSSON and LARSSON 1961 ANDERSSON *et al* 1963) Controlled central cooling could in this manner be carried out for long periods of time while the goats were maintained with no additional restraint in their normal environment. The method of studying thyroidal activity was that used in previous studies (ANDERSSON *et al* 1962 a, b c 1963)

In the present series of experiments the periods of central cooling in each animal were always of the same duration (3 hours in goat A and 2 hours in goat B) The degree of central cooling was in both animals maintained at the same level at all periods.

Exogenous thyroidal hormone used to study the feed back effect was Thyroxine "Roche", and was administered intravenously into the jugular vein

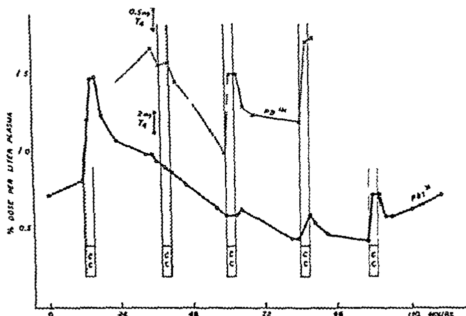


Fig 1 *Suppression of the thyroidal response to cooling of the heat loss center by injection of thyroxine*

Lower curve The first 3 hour period of central cooling (cc) evoked the usual conspicuous rise in plasma PBI[131] The response to identical cooling the following day performed 2 hours after the i.v injection of 2 mg of thyroxine (T_4) was completely blocked It gradually reappeared 48 to 72 hours later Note that the depression of the plasma PBI[131] curve after the injection of this large dose of thyroxine continues during the first post injection period of central cooling and lasts for about 3 days

Upper curve A similar but more short lasting blockade obtained by the i v injection of 0 5 mg of thyroxine (T)

Results

During the first series of experiments in goat A preoptic/anterior hypothalamic cooling was initially performed 60 hours after the administration of radio iodine (i e during the rising phase of plasma protein bound iodine (PBI[131])) During this period of central cooling (not preceded by any injection of thyroxine) the plasma PBI[131] rapidly increased by 80 % of the pre cooling value On the following day 2 mg of thyroxine was given i v to the animal The administration of thyroxine rapidly induced a gradual decline of the plasma PBI[131] level which continued for 3 days Central cooling performed 2 hours after the thyroxine was given caused no rise in the plasma PBI[131] rather the level continued to fall even during central cooling A repeated period of central cooling 24 hours later caused very little change in the plasma PBI[131] However 48 hours after the administration of thyroxine central cooling caused some thyroid activation and a more pronounced response was obtained the following day (Fig 1 lower curve) Thyroxine in a dose of 55 μg/kg b wt thus in this animal depressed the

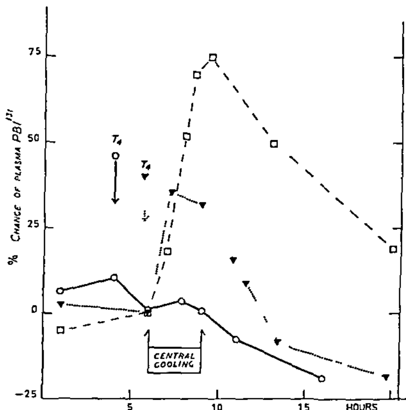


Fig 2 *Three experiments in the same animal to show the gradual development of thyroxine inhibition of the thyroidal response to local cooling of the heat loss center*

Open squares The normal elevation of plasma PBI obtained by central cooling
Open circles In contrast the i v injection of thyroxine (T_4 about 15 μg/kg b wt) completely blocked the thyroidal response to central cooling performed two hours later
Solid triangles The partial blockage obtained when the same amount of thyroxine (T_4) was injected only 20 min prior to central cooling Note that the plasma PBI level here starts to fall again before termination of central cooling

normal release of hormone from the thyroid for at least 3 days and completely inhibited the thyroidal response to local cooling of the heat loss center for at least 24 hours

Three weeks later another series of experiments were performed in parallel in both animals The doses of thyroxine used to study the feed back control were then limited to 0 5 mg In goat A this dose of thyroxine was given i v on the third day after radioiodine administration and central cooling was started 2 hours later This period of central cooling caused no thyroid activation Renewed central cooling performed 24 and 48 hours later were however, effective in this respect (Fig 1 upper curve) Similar results were obtained in parallel experiments in goat B Thus the i v administration of thyroxine in a dose of about 15 μg/kg b wt within 2 hours blocked in these animals the thyroi

dal response to local cooling of the "heat loss center The blocking action however of this small dose of thyroxine lasted for less than 24 hours

It was further decided to study whether the time interval between the administration of thyroxine and the onset of central cooling was of importance for the development of the blocking action Therefore in other experiments central cooling was started as early as 15 (goat B) and 20 (goat A) min after the i v injection of 0 5 mg of thyroxine Despite the fact that the general blood level of thyroxine at this time could be expected to be higher than 2 hours after the administration of hormone central cooling in both animals caused marked thyroid activation The thyroid response however was not so pronounced as that obtained when central cooling was performed without previous injection of exogenous thyroxine (Fig 2)

The blocking action of thyroxine thus seemed to develop gradually during the first hours after the i v injection of thyroxine

Discussion

The use of I^{131} labelled thyroxine has revealed that this hormone is highly concentrated in the median eminence region and in the neurohypophysis whereas its concentration in the adenohypophysis is small (JENSEN and CLARK 1951 COURRIER *et al* 1951 GROSS and PITT RIVERS 1952 and others) This accumulation of thyroid hormone in the median eminence region has led BROWN GRANT (1957) to suggest that the hypothalamus may act as a filter for thyroxine so that the blood reaching the anterior pituitary via the hypothalamo-hypophyseal portal vessels has a lower hormonal concentration than that of the general circulation Factors changing the extraction rate of thyroxine in the hypothalamus would then cause local variations in the hormone concentration of blood reaching the adenohypophysis resulting in increased or decreased TSH secretion BROWN GRANT s hypothesis thus provides a possible explanation of nervous system control of thyroid function

According to BROWN GRANT the thyroid stimulating effect of cold may also be explained on the basis of the negative feed back control of TSH secretion exerted by thyroxine In the cold peripheral utilization or disposal of thyroxine is accelerated (DEMPSEY and ASTWOOD 1943) and an increased release of thyroid hormone is generally not obtained until 4 to 8 hours after beginning of cold exposure A lowered level of circulating thyroxine may therefore be the proximate cause of increased TSH release and thyroid activation in the cold

Local cooling of the preoptic/anterior hypothalamic heat loss center has recently been shown to be a much more effective and rapid way to induce thyroid activation than exposure to external cold (ANDERSSON *et al* 1962 b 1963) Increased release of thyroid hormone is thus generally observed one half to one hour after the onset of central cooling *i e* almost as soon as after an

i v injection of TSH, and relatively short periods of central cooling give a highly reproducible response when repeated at 24 hour intervals Since local warming of the heat loss center has the opposite thyroidal effect it has been suggested that "warm detectors in this region exert a tonic inhibition on the release of TSH from the adenohypophysis A complete removal of such an inhibition would then be the ultimate cause of the conspicuous thyroid activation obtained by local cooling of the heat loss center

The present study has unequivocally shown that thyroxine feed back inhibition of TSH secretion blocks the thyroid response to local cooling of the heat loss center It thus seems certain that the thyroidal effect of cooling this center is really mediated via the hypothalamic pituitary thyroid axis Local cooling of the preoptic/anterior hypothalamic region therefore may be used in the future with advantage for a more detailed analysis of the feed back control of TSH secretion

The difference in thyroidal response to cooling the heat loss center 20 min and 2 hours after the i v injections of relatively small amounts of thyroxine (Fig 2) is of particular interest Central cooling performed 20 min after such an injection still caused marked thyroid activation whereas no response was obtained by cooling 2 hours after the administration of thyroxine In the first instance the level of thyroxine in the general circulation must certainly have been higher than in the second instance For this reason it seems most unlikely that a lowering of the level of circulating thyroxine, caused by increased peripheral utilization should be the proximate cause of TSH release during central cooling The rapidity of the thyroidal response to cooling the heat loss center also speaks against this possibility

The delay time before a complete blockage developed further suggests that an accumulation of thyroxine has to take place at some site before the release of TSH is completely inhibited A gradual saturation of a median eminence filtering off mechanism of the kind suggested by BROWN GRANT (1957) may perhaps be the explanation of this phenomenon

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Smooth Muscle Phosphorylase and Enzymes Affecting its Activity

By

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Abstract

Mohme Lundholm E *Smooth muscle phosphorylase and enzymes affecting its activity* Acta physiol scand 1963 59 74—84 — The activity per mg protein of purified phosphorylase *a* prepared from smooth muscle (bovine tracheal muscle) was found to be approximately one half that of the corresponding purified enzyme from striated muscle Under the action of phosphorylase phosphatase (PR enzyme) from tracheal or skeletal muscle phosphorylase *a* was converted to phosphorylase *b* coincidently with liberation of inorganic phosphate A higher concentration of AMP was required for activation of smooth muscle phosphorylase *b* than for activation of striated muscle phosphorylase *b* Partially purified phosphorylase phosphatase from bovine tracheal muscle had per mg protein approximately one fourth the activity of the corresponding enzyme from rabbit skeletal muscle In the presence of ATP and magnesium ions phosphorylase *a* was synthetized from phosphorylase *b* by phosphorylase *b* kinase prepared from tracheal or skeletal muscle Inactive phosphorylase *b* kinase was activated by adenosine 3′—5′ phosphate by calcium ions and by increasing the pH of the incubation solution to 8.4

In a previously reported study adrenaline was found to have a stimulatory effect on the phosphorylase activity in smooth muscle (Mohme Lundholm 1962) In striated muscle adrenaline activates phosphorylase by stimulating the synthesis of cyclic 3′—5′ adenosine monophosphate (3′—5′ AMP) (For review vide Sutherland and Rall 1960) This nucleotide in the presence of ATP and magnesium ions activates the enzyme phosphorylase *b* kinase which in turn in the presence of ATP and magnesium ions promotes the synthesis

of phosphorylase *a* from phosphorylase *b* Phosphorylase *b* kinase can be isolated from muscle in a form that is inactive below pH 7 This form is activated by cyclic 3'—5' AMP calcium ions and an increase of pH (KREBS GRAVES and FISCHER 1959) Phosphorylase *a* is converted into phosphorylase *b* by the enzyme phosphorylase phosphatase (PR enzyme) whereby inorganic phosphate is split off (KREBS *et al* 1958)

The question remains whether adrenaline in smooth muscle activates phosphorylase via the same mechanism that applies to striated muscle Cyclic 3'—5' AMP has been demonstrated in smooth muscle but there are no indications that adrenaline stimulates its synthesis in smooth muscle (KLAINER *et al* 1962) Nor is there any conclusive evidence of the existence of phosphorylase *b* kinase and phosphorylase phosphatase activity in smooth muscle Lastly it has not been established whether in smooth muscle phosphorylase occurs as the *a* form (active in the absence of AMP) and the *b* form (active only in the presence of AMP) or whether as in the liver it occurs in an active and an inactive form the activity of which is not affected by AMP According to AXELSSON BUEDING and BÜLBRING (1961) the phosphorylase activity in crude extract of *taenia coli* from guinea pig does not increase on addition of AMP even though the total phosphorylase activity shows appreciable variations These results suggest that in smooth muscle as in the liver both an active and an inactive form of phosphorylase occur Yet MOHME LUNDHOLM (1962) found that in crude extracts from bovine tracheal muscle rabbit gut rabbit stomach and rat aorta AMP served to increase the phosphorylase activity by as much as 50 per cent — a finding which suggests that both the *a* and the *b* form of phosphorylase may have been present in those organs

Reported in the following are experiments which demonstrate that the *a* and *b* forms of phosphorylase occur in smooth muscle (bovine tracheal muscle) and which indicate that the enzyme affecting the phosphorylase activity are identical with those which influence that activity in striated muscle (rabbit skeletal muscle) Inactive phosphorylase *b* kinase from tracheal muscle can be activated by calcium ions cyclic 3'—5' AMP and an increase of pH

Methods

Phosphorylase *a* was prepared and its activity determined in accordance with a method reported by CORI ILLINGWORTH and KELLER (1955) The tissues used for extraction were bovine tracheal muscle and rabbit skeletal muscle In the case of tracheal muscle a greater volume of water than the recommended amount was used for extraction since the quantities of tissue available were relatively small and the additional water increased the extractable amount of phosphorylase *a* (MOHME LUNDHOLM 1962)

Phosphorylase *b* was prepared *ad modum* CORI and CORI (1945) through the action of phosphorylase phosphatase upon phosphorylase *a* after which the phosphorylase phosphatase was inhibited by addition of 0 02 M sodium fluoride Direct preparation from muscle extract *ad modum* FISCHER and KREBS (1958) was also tried Phosphorylase *b* activity was determined in the presence of 0 001 M AMP

Table I Preparation of phosphorylase a from bovine tracheal muscle and from rabbit skeletal muscle From 1 g tracheal muscle 223 units phosphorylase was extracted and from skeletal muscle 675 units per g Determined with AMP

Step of preparation	Tracheal muscle 12 g/50 ml			Skeletal muscle 450 g/600 ml		
	Protein mg per ml	Units a per mg protein	Units + + AMP per mg protein	Protein mg per ml	Units a per mg protein	Units + AMP per mg protein
1 After extraction centrifu gation filtration	48 5	1 1	1 2	22 6	17 8	22 8
2 pH to 6 4 dialysis pH to 6 0 centrifugation filtra tion pH to 6 8	42 0	4 4	4 7	21 2	14 4	15 4
3 Precipitation with $(NH_4)_2SO_4$ centrifugation dis-*solved in* H_2O	29 4	*17 1*	*19 8*	*56 2*	*214 3*	*231 1*
4 Dialysis H_2O dialysis a gainst cysteine-glycerophos-phate solution centrifu gation, recrystallization	10 0	913	1 002	9 1	1 720	1 810
5 Third recrystallization No rit treatment	7 1	1 333	1 474	—	—	—

Phosphorylase phosphatase (PR enzyme) was prepared by the method of KELLER CORI (1955)

For preparation of phosphorylase *b* kinase in inactive form the method of KREBS GRAVES and FISCHER (1959) — precipitation with acetic acid at pH 5 8 — was employed It was unfortunately impossible to determine the phosphorylase *b* kinase activity in units *ad modum* KREBS and FISCHER (1956) since I have not yet succeeded in extracting from tracheal muscle a sufficiently high concentration or a sufficient amount of phosphorylase *b* to permit such determination Instead a more qualitative mode of determination was applied as follows Of a solution containing 15—20 units phosphorylase *b* per ml in 0 015 M neutral cysteine 2 ml was incubated with 1 ml 0 25 M tris + 0 25 M sodium glycerophosphate buffer the pH being adjusted to 6 8 or 8 1 1 ml of a solution containing 6 10 $^{-2}$M magnesium acetate and 1 8 10 $^{-2}$M ATP 1 ml phosphorylase *b* kinase extract (undiluted) and 1 ml 5 10^{-3}M calcium acetate or 1 ml 1 10^{-4}M cyclic 3 —5 AMP or 1 ml water (control) Following incubation 0 8 ml of this solution was taken and 0 001 M EDTA added in order to terminate the reaction after which the phosphorylase activity was determined

Phosphorylase activity was determined *ad modum* CORI ILLINGWORTH and KELLER (1955) at pH 6 8 and with 0 1 M sodium fluoride for inhibition of any phosphoglucomutase activity The incubation time was generally 10 min whereby 1—15 per cent of the total phosphorus in glucose 1 phosphate was liberated Inorganic P was determined by the method of MARTIN and DOTY (1949)

For protein determination the procedure of LOWRY *et al* (1951) was employed

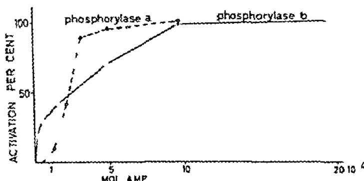


Fig 1 Activation of tracheal muscle phosphorylase *a* and *b* by AMP at various concentrations The phosphorylase *a* activity in the absence of AMP was 85 per cent of that in the presence of 0 001 M AMP the range of activation thus being 85—100 per cent In the experiments with phosphorylase *b* the corresponding range was 15—100 per cent

AMP was determined by an enzymatic method in accordance with BUCHER s (1953) principle and the directions given for the Boehringer (Mannheim) enzymatic ADP/AMP test

Glucose 1 phosphate ATP AMP and cyclic 3 —5 AMP were obtained from Sigma Glycogen (Merck) was purified by ion exchange chromatography as described earlier (MOHME LUNDHOLM 1962)

Results

Phosphorylase a

In Table I the mode of preparation of phosphorylase *a* from bovine tracheal muscle and from rabbit skeletal muscle is schematized It will be seen that after the third recrystallization the tracheal muscle phosphorylase activity per mg protein (1 333 units) approximated the corresponding skeletal muscle activity (1 720 units/mg protein) after one crystallization CORI and CORI (1945) have found a maximal skeletal muscle phosphorylase *a* activity of about 3 000 units/mg protein

According to GREEN and CORI (1943) AMP also influences rabbit muscle phosphorylase *a* activity In the absence of AMP the phosphorylase *a* activity was only 65 per cent of the activity determined in its presence AMP at a concentration of 1 5 10^{-4}M produced a 50 per cent activation — counting between 65 and 100 per cent activity — of phosphorylase *a* There appeared to be a certain disparity between phosphorylase *a* from rabbit skeletal muscle and that from tracheal muscle In three different preparations treated with activated charcoal to remove AMP the tracheal muscle phosphorylase *a* activity (without AMP) ranged from 85 to 90 per cent of that determined in the presence of AMP Since however AMP seems to have an appreciable affinity for phosphorylase *a* (MADSEN and CORI 1957) the possibility that tracheal muscle phosphorylase was not entirely free from AMP could not be [illegible]

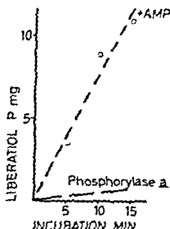


Fig 2 Determination of the activity of phosphorylase *b* (from tracheal muscle) prepared according to FISCHER and KREBS (1958) with and without 0 001 M AMP The solution contained 0 45 mg protein per 1 ml, equivalent to 25 units phosphorylase *b* per mg and 1 2 units phosphorylase *a* per mg

The AMP content of 10 mg phosphorylase *a* which had been treated with activated charcoal was therefore determined by an enzymatic method capable of disclosing amounts down to 2 μg per test No AMP was demonstrable however, in perchloric acid extracts from phosphorylase *a*

On determination of the relationship between activation of phosphorylase *a* from tracheal muscle and the AMP concentration no activating effect was demonstrable until the concentration reached approximately 1 10^{-4}M AMP, and at 2 3 10^{-4}M AMP the activation was half maximum (Fig 1)

It thus emerged that phosphorylase *a* from smooth muscle was less than one hundredth as sensitive to the activating effect of AMP as was the corresponding enzyme from rabbit skeletal muscle Since the AMP concentration producing 50 per cent activation of phosphorylase *a* from tracheal muscle was equal to that which produced a corresponding activation of phosphorylase *b* (Fig 1) the question arises whether activation of the *a* form was caused by contamination by the *b* form

It was sought to determine the homogeneity of phosphorylase *a* by means of analytical ultracentrifugation In 0 03 M glycerophosphate buffer plus 0 03 M cysteine however the enzyme proved to be only about 0 2 per cent soluble which was insufficient for the purpose When an attempt was made to enhance the solubility by increasing the ionic strength with 0 1 M KCl the enzymatic activity disappeared and that agent was therefore unsuitable

Phosphorylase b

Two procedures were employed for preparations of phosphorylase *b* from tracheal muscle The first consisted in incubation of phosphorylase *a* with phosphorylase phosphatase from tracheal or skeletal muscle This phosphorylase phosphatase had been treated with activated charcoal for removal of AMP After 10 hours incubation the phosphorylase activity in the absence of

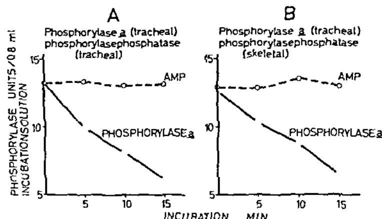


Fig 3 Three ml of phosphorylase *a* containing 20 μg enzyme protein per 1 ml plus 3 ml phosphorylase phosphatase from tracheal muscle (1 96 mg protein per 1 ml) or from skeletal muscle (0 225 mg protein per 1 ml) was incubated at 30 C After 5 10 and 15 min incubation 0 8 ml was taken for determination of the phosphorylase activity *ad modum* CORI and ILLINGWORTH (1955) with and without 0 001 M AMP

Table II The activity of tracheal and skeletal muscle phosphorylase phosphatase on phosphorylase a from tracheal and skeletal muscle

Substrate	Tracheal phosphorylase phosphatase		Skeletal phosphorylase phosphatase	
	Units per mg protein	Liberated P μM per units *a*	Units per mg protein	Liberated P μM per units *a*
Tracheal muscle phosphorylase *a*	28 4	0.81	104 0	0 77
Skeletal muscle phosphorylase *a*	34 4	0 61	147 3	0 59

AMP had decreased to 15 per cent of that occurring in the presence of AMP (Fig 5) It was sought to precipitate phosphorylase *b* by dialyzing it against 30 per cent ammonium sulphate solution then against 0 03 M sodium β glycerophosphatecysteine buffer according to CORI and CORI (1945) Under this treatment however the phosphorylase activity diminished appreciably Whereas the original solution contained about 1 000 units per mg of protein the activity declined to approximately 100 units/mg after precipitation and dialysis

It was decided therefore to utilize the method of FISCHER and KREBS (1958) for direct extraction of phosphorylase *b* from tracheal muscle In an extract thus prepared approximately 95 per cent of the phosphorylase activity was attributable to phosphorylase *b* (Fig 2) However the activity amounted at

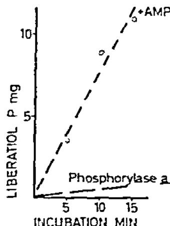


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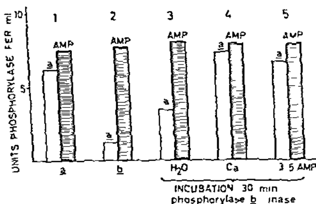


Fig 5 Inactivation and reactivation of tracheal muscle phosphorylase *a* with phosphorylase phosphatase and activated phosphorylase *b* kinase from tracheal muscle 1 Activity of phosphorylase *a* without AMP and with 0 001 M AMP 2 After 10 hours incubation with phosphorylase phosphatase 3 After 30 min incubation of *b* with phosphorylase *b* kinase at pH 6 9 4 After incubation with phosphorylase *b* kinase plus 8 10^{-4} M Ca^{++} 5 After incubation with phosphorylase *b* kinase plus 1 7 10^{-4} M adenosine 3—5 phosphate

Under the action of phosphorylase phosphatase upon phosphorylase *a* in organic phosphate was split off On the average 0 60 μmole phosphate was liberated for each unit of skeletal muscle phosphorylase *a* split off (Table II) For tracheal muscle phosphorylase *a* the amount of phosphate liberated was approximately 30 per cent higher or 0 79 μmole per unit This may suggest that with the splitting of 1 mole tracheal muscle phosphorylase *a*, 5 moles rather than 4 moles phosphate is liberated

Phosphorylase b kinase

It was found as noted by KREBS GRAVES and FISCHER (1959) that phosphorylase *b* kinase could be isolated from skeletal muscle in an inactive form which was activated by calcium ions and cyclic 3—5 AMP thereby synthesizing skeletal muscle phosphorylase *a* from phosphorylase *b* — prepared *ad modum* FISCHER and KREBS (1958) — in the presence of ATP and magnesium ions (Fig 4) With the same method it was also possible to isolate inactive phosphorylase *b* kinase from tracheal muscle This enzyme was activated by calcium ions and cyclic 3 – 5 AMP (Fig 5) and by an increase of the incubation solution pH to 8 4 (Fig 6) Phosphorylase *a* was synthesized from tracheal muscle phosphorylase *b* Fig 5 illustrates an experiment in which tracheal muscle phosphorylase *a* was converted to phosphorylase *b* by phosphorylase phosphatase The *b* form was thereafter resynthesized to *a* by activated phosphorylase *b* kinase Further experiments were performed with phosphorylase *b* prepared directly from tracheal muscle *ad modum* FISCHER and KREBS (1958) Phosphorylase *a* was synthesized even from this *b* form by phosphorylase *b* kinase

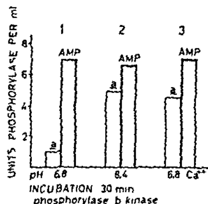


Fig 6 Tracheal muscle phosphorylase *b* after 30 min incubation with phosphorylase *b* kinase from tracheal muscle 1 At pH 6 8 2 At pH 8 4 3 At pH 6 8 plus 8 10^{-4} M Ca^{++}

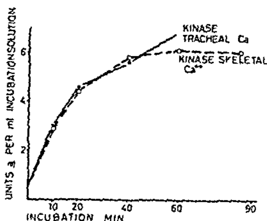


Fig 7 Reaction velocity in the synthesis of phosphorylase *a* from *b* (from skeletal muscle) under the action of Ca^{++} activated phosphorylase *b* kinase (from tracheal and skeletal muscle)

There was no major disparity between the activity of phosphorylase *b* kinase from tracheal muscle and that from skeletal muscle It is evident from Fig 7 that the synthesis of skeletal muscle phosphorylase *a* from *b* proceeded at the same rate irrespective of whether the calcium ion activated phosphorylase *b* kinase used was from tracheal or from skeletal muscle

The possibility existed that the intensification of phosphorylase activity by phosphorylase *b* kinase was due to the presence of AMP in the extract or to its formation during incubation with ATP Determination of the AMP content of extracts containing phosphorylase *b* kinase gave a value of 30 μg per ml After incubation with ATP and and calcium ions the content was 25 μg per ml The AMP content of the phosphorylase *b* kinase extracts was estimated to have raised the AMP concentration in the phosphorylase *b* tests by 0 7 10^{-5}M (the dilution was 1 7 5) It may be calculated from Fig 1 that phosphorylase *b* thereby underwent 5 per cent activation Thus an elevation of the AMP concentration cannot account for the total increase of phosphorylase activity after phosphorylase *b* kinase

Discussion

This comparative investigation of phosphorylase and the enzymes which influence its activity suggests that the enzymatic patterns in bovine tracheal muscle and rabbit skeletal muscle are essentially similar Both the *a* and the *b* forms of phosphorylase were isolated from each of the two types of muscle as were phosphorylase phosphatase and phosphorylase *b* kinase Phosphorylase and phosphorylase phosphatase from smooth muscle had, per mg of protein a lower activity than the corresponding enzymes from striated muscle Whether this was due to differing degrees of purification or whether it reflected a genuine discrepancy is an open question It would seem however that higher concentrations of AMP were required for activation of smooth muscle phosphorylase than for activation of skeletal muscle phosphorylase

Thus despite conspicuous qualitative similarities substantial quantitative differences emerged The phosphorylase activity in crude extracts from tracheal muscle was only about one tenth of that in extracts obtained from skeletal muscle by the same procedure (MOHME LUNDHOLM 1962) Furthermore the bulk of tracheal muscle phosphorylase was the *a* form whereas the *b* form predominated in skeletal muscle phosphorylase The question arises whether in tracheal muscle the synthesis of phosphorylase *a* was increased or the breakdown decreased in relation to the behaviour in skeletal muscle The lower activity of tracheal muscle phosphorylase phosphatase per mg of protein is possibly suggestive of a lower breakdown in tracheal muscle particularly in view of the fact that there was no appreciable difference between the amounts of calcium ion activated phosphorylase *b* kinase from respectively tracheal and skeletal muscle

Phosphorylase *b* kinase from tracheal muscle was activated by cyclic 3 —5 AMP in the presence of ATP and magnesium ions Biochemically the possibility exists therefore that in tracheal muscle as in skeletal muscle adrenaline may stimulate the formation of phosphorylase *a* via cyclic 3 —5 AMP KLAINER *et al* (1962) have however failed to demonstrate any significant stimulation of the synthesis of cyclic 3 —5 AMP in smooth muscle after adrenaline LUNDHOLM and MOHME LUNDHOLM (1962) have found that adrenaline increased the ATP content of smooth muscle As ATP participate in several steps in the formation of phosphorylase *a* adrenaline might influence the phosphorylase activity by an increased ATP formation

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Mechanics of Abdomen and Thorax in Man during Natural Breathing

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Abstract

NISELL, O *Mechanics of abdomen and thorax in man during natural breathing* Acta physiol scand 1963 *59* 85—96 — The intra gastric pressure fluctuation during natural breathing is presumed to indicate the pressure moving the abdomen and thoracic wall The mechanics of the abdomen and thoracic wall were estimated in resting human subjects by means of intra gastric pressure tidal volume curves Compliance was 0 06—0 23 l/cm water resistance 0 5—6 5 cm water/l/sec and rate of inspiratory work 15—100 litre cm water/min (100 litre cm water = 1 kpm) for abdomen and thoracic wall together Supine as compared to sitting position increased the compliance and decreased the resistance The abdomen was calculated to have a higher compliance and a lower resistance than the thoracic wall and to account for more than 0 6 of resting ventilation

In certain clinical and physiological ventilatory studies it is necessary to consider the mechanics of the whole of the respiratory system Our konwledge of the characteristics of the lungs and airways is fairly extensive whereas relatively little is known with regard to the thorax and abdomen For studying these outer parts of the respiratory apparatus in man voluntary relaxation (RAHN *et al* 1946 OTIS *et al* 1950 AGOSTONI and RAHN 1960 MEAD 1960) anesthesia (NIMS *et al* 1955 BUTLER and SMITH 1957 HOWELL and PECKETT 1957 BROMAGE 1958) displacement of respiratory level HEAF and PRIME 1956 DAHLSTROM 1959 NADMARK and CHERNIACK 1960) and oscillation of the chest (DUBOIS *et al* 1956 MEAD 1960) have been utilized Each of these procedures however permits a study only of certain components of respiratory mechanics In the present paper several aspects of abdominal and thoracic mechanics are evaluated simultaneously by a different approach

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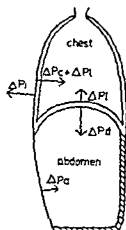


Fig 1 Respiratory tension change in intercostal muscles (Δ Pi) diaphragm (Δ Pd) chest wall (Δ Pc) lungs (Δ Pl) and abdominal wall and tissue (Δ Pa) The intra abdominal (intra gastric) pressure change is not indicated but of the same magnitude as Δ Pa (and directed against this and Δ Pd) It is presumed that during natural breathing Δ Pd = Δ Pi Δ Pd = Δ Pa + Δ Pl Δ Pi = Δ Pc + Δ Pl and Δ Pa = Δ Pc = intra-gastric pressure change

Methods and Subjects

Records have been made of the respiratory pressure fluctuations within the oesophagus and the stomach They were obtained via a Porges neoplex duodenal catheter of 2 mm inside diameter with one end inserted into a thin walled latex balloon about 12 cm long and 10 mm in diameter The balloon in air tight communication with the catheter was passed through the nose to the lower part of the oesophagus until the catheter tip was approximately 45 cm from the nostril and then further until the tip was about 65 cm from the nostril and the balloon completely in the stomach Pressure was measured with a strain gauge or differential electromechanical manometer (EMT 490 BD NO B 289 A. B Elema Sweden) The difference between the pressure in the oesophagus and that in the mouth piece was registered and used in the calculations of the lung mechanics Intra gastric pressure was recorded against atmo phere pressure The balloon manometer system was filled with 2 ml air starting at approximately —10 cm H_2O for oesophageal measurements and at zero pressure for intra-gastric pressure studies

The subject with nose clamped breathed through a mouth piece into an air filled closed circuit Krogh spirometer There was no change of amplitude and no appreciable phase shift between pressure and volume recordings up to the frequency of one per sec

The curves were recorded directly as pressure volume loops with an Elema coordinate ink writer (NISELL *et al* 1958) The movements of the spirometer determined mechanically the vertical deflections of the writer and the electric pressure impulses governed the horizontal deflections The ink jet was interrupted 6 times per sec to time mark the curves Unless otherwise stated a minimum of ten respirations was recorded for each position and each procedure They are represented below by their mean values

The intra-oesophageal pressure tidal volume loop is well known and was used to derive the lung mechanics The intra gastric pressure tidal volume curve may be used in a similar way to indicate the mechanics of the abdomen and thoracic wall During ordinary inspiration the lower intercostal muscles as well as the diaphragm contract The two muscle groups may be presumed to tense *equally* during inspiration The intra gastric pressure fluctuations will then represent the pressure which moves the abdomen and thoracic wall provided that lung tension changes at the diaphragm in the same degree as at the thoracic wall and that expiration is passive (cf Fig 1) The compliance and resistance of abdomen and thoracic wall together as well as the respiratory work pertaining to them, can be calculated when tidal volume respiratory flow and intra gastric respiratory pressure fluctuations are known

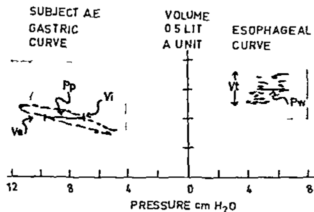


Fig 2 Tidal volume recorded against intra-oesophageal pressure (esophageal curve) and intra gastric pressure (gastric curve) in subject 3 Inspiration starts at lowest point of curve Esophageal curve written counter-clockwise and gastric curve clockwise Resistance measured (see esophageal curve) from PwT/2πVt where Pw is maximum width of loop at one volume Vt tidal volume and T duration of breath but sometimes (see gastric curve) from Pp Tp/(Vi + Ve) where Pp is pressure between two pairs of points at same volume Tp time between two consecutive points and Ve and Vi volume changes in each pair (NISELL and EHRNER 1958)

Compliance was obtained from volume change/pressure change between onset and termination of inspiration or expiration except at very slow breathing when the relationship was represented by the slope of the curve Resistance was ordinarily calculated as indicated on the oesophageal curve in Fig 2 utilizing the maximum width of the curve at one volume and assuming respiratory flow to be approximately sinusoidal (NISELL and EHRNER 1958) The resistance in such cases referred to maximum flow during a respiration In some instances mostly during artificial ventilation resistance was calculated as indicated on the gastric curve in Fig 2 from curve width and instantaneously measured respiratory flow Resistive respiratory work was computed from the loop area measured with a planimeter and inspiratory work was the sum of the elastic work performed during inspiration and half the resistive work Work per respiration was multiplied by respiratory frequency to obtain the rate of respiratory work Resistance and respiratory work values for lungs added to those for abdomen and thoracic wall during natural breathing gave the corresponding values for the total respiratory system Similarly total compliance (C_m) was derived from compliance of lungs (C_l) and compliance of abdomen and thoracic wall (C) according to $1/C_m = 1/C_l +$ 1/C Volumes were corrected to BTPS

Using a circuit analogy abdomen and thorax are ventilated as connected in parallel and the tidal air volume is the sum of their respiratory volume displacements The physical properties of abdomen and thorax together determine the magnitude of the mechanical characteristics calculable from the intra gastric pressure tidal volume curve during natural breathing If the subject s breathing is purely diaphragmatic the abdomen alone should account for the compliance and resistance values obtained When the compliance and resistance of the abdomen C and R and of abdomen and thorax together C_t and R are known it should be possible to calculate the compliance and resistance of the thoracic wall C and R_t from $C_t = C - C$ and $1/R_t = 1/R_t - 1/R$ The abdominal fraction of natural breathing was estimated from the relation of the impedance at natural breathing and the impedance at abdominal breathing The impedance was the ratio of intra gastric pressure amplitude to maximum airflow during a respiration (NISELL 1960) For the purpose of these examinations I instructed some of the subjects intermittently to concentrate on abdominal breathing alone and watched them to make sure that they did so as well as possible

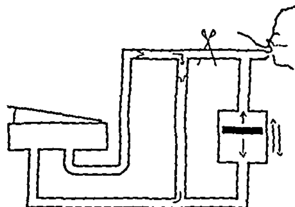


Fig. 3. Principle of spirometer subject connection during artificial ventilation. Clamp open: subject breathes spontaneously. Clamp closed: subject relaxes and is artificially ventilated by continuously moving piston. Pressure registered in mouth piece or oesophagus.

Further expiration from functional residual capacity is effectuated by intercostal and abdominal muscles. These two muscle groups may then be assumed to elicit equal tension. During very slow excessive expiration the muscle force indicated by the abdominal pressure is balanced by elastic recoil of the thoracic wall, diaphragm and lungs. The compliance of the thoracic wall, diaphragm and lungs together should then be represented by the volume pressure relationship of the corresponding part of the recorded intra gastric pressure tidal volume curve.

Artificial respiration was given to some subjects as shown in Fig. 3. The pump was operated at a stroke volume of 0.6 litre and a frequency of 20/min. The subject breathed spontaneously when the clamp was open. He was instructed to relax when the clamp was closed and not to facilitate or impede the ventilation from the continuously moving piston. Mouth piece or oesophageal pressure and spirometer deflection were then registered as pressure volume diagrams. Compliance and resistance were calculated for the total respiratory apparatus (mouth piece pressure) or for the thoracic wall abdomen (oesophageal pressure). Only a limited number (3—14) of usable curves were obtained in each study.

The subjects were studied in the seated and sometimes in the recumbent position. The normal subjects except subject I G were medical students and the others were patients at the hospital. Table I gives the sex, age, height, weight and diagnosis.

Results

The magnitude of the respiratory pressure fluctuation and end expiratory pressure in the stomach will be seen in Table II. The sum of the intra gastric and intra oesophageal pressure fluctuations during a respiration is the trans diaphragmatic pressure fluctuation. The balloon air volume affected the gastric pressure as shown in Fig. 4. The pressure decreased when the air volume was below approximately 1.5 ml.

The conventional form of the volume/pressure curves when the balloon is in the oesophagus (oesophageal curve) and in the stomach (gastric curve) are shown in Fig. 2. The gastric curves of four subjects transiently exhibited a small extra loop at the end of expiration and at the beginning of inspiration during

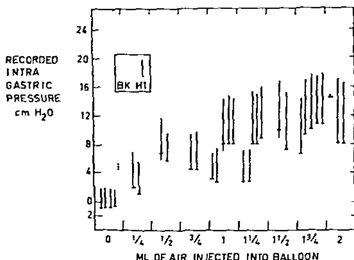


Fig 4 Relation of air volume in gastric balloon and pressure fluctuation in two subjects Each vertical line represents a recorded breath Short horizontal cross line indicates end expiratory pressure Subject 16 and 17 (A)

Table I Physical characteristics and diagnosis of subjects

Subject	Sex	Age (years)	Height (cm)	Weight (kg)	Note
1 L. A	♀	28	169	57	Normal
2 I G	♀	18	157	45	Normal
3 A. E.	♂	30	183	70	Normal
4 P M	♂	28	184	63	Normal
5 M N	♂	24	175	71	Normal
6 S. K	♂	39	179	64	No present pathology
7 O C.	♂	28	170	76	No present pathology
8 J F	♂	25	174	54	No present pathology
9 E. J	♀	17	171	55	Rheumatic fever
10 O O	♂	26	177	69	Vagal neurosis
11 S B	♀	67	163	65	Pulmonary tuberculosis
12 K. B	♂	59	174	95	Emphysema
13 E. H	♂	61	177	72	Emphysema
14 J P	♂	57	169	55	Emphysema
15 V S	♂	56	168	63	Emphysema
16 B K	♂	58	172	62	Emphysema
17A H I	♂	43	171	60	Costal fractures
17B H I	♂	43	171	60	Costal fractures
18 L. B	♂	29	183	70	Costal fractures

17A and 17B are two studies of one subject B 45 days after A.

natural breathing Such curves were not used to estimate given values The gastric curves of five out of eight subjects took this form during intentional hyper ventilation (Fig 5)

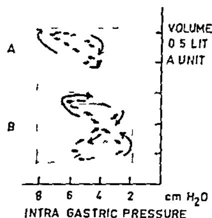


Fig 5 Gastric curves with figure-of 8 loop Inspiration upwards A Subject 15 natural breathing B Subject 18 hyperventilation

Compliance resistance and rate of respiratory work for the abdomen and thoracic wall and for the total respiratory system are presented in Table II The range of the values for the abdomen and thoracic wall is about the same in subjects with and without disease

The breath to breath variability of the characteristics was estimated The coefficient of variation calculated in each subject averaged during natural breathing for respiratory pressure fluctuations 15 2 per cent in curves of the oesophagus (standard deviation 8 0) and 15 3 per cent in curves of the stomach (standard deviation 10 6) The coefficients of variation for the abdomen and thoracic wall averaged 17 7 per cent for compliance 22 9 per cent for resistance and 23 4 per cent for work of breathing with standard deviation of 7 6 9 3 and 8 1 per cent respectively

Subject 9 was wearing a girdle when the values in Table II were obtained Following removal of the girdle in supine position the compliance of abdomen and thoracic wall increased from 0 12 to 0 20 litre/cm water while the resistance decreased from 3 6 to 2 4 cm water/litre/sec

Intra gastric pressure fluctuation compliance resistance and rate of respiratory work for abdomen and thorax were determined in 7 subjects both in sitting and in recumbent posture Compliance was higher and resistance intra gastric pressure amplitude and rate of respiratory work lower in the supine than in the sitting position (Table III)

Gastric curves were registered during abdominal and natural breathing in 1 female and 7 male subjects The values for abdominal breathing as well as those estimated for the thoracic wall are given in Table IV The compliance of the abdomen was invariably greater than that calculated for the thoracic wall Abdominal resistance was lower than thoracic resistance in 6 cases The abdominal fraction of ventilation calculated from the impedance values of natural and diaphragmatic breathing appears to be above 0 6

Table II Mechanics of the abdomen and thoracic wall and the total respiratory system during natural breathing

Subject	Respiratory pressure fluctuation (cm H O)		End-expiratory pressure (cm H O)		Compliance (l/cm H O)		Resistance (cm H O/l/sec)		Rate of inspiratory work (l cm H O/min)	
	Intra gastric	T d.	Intra gastric	T -d.	Abdomen thor wall	Total	Abdomen thor wall	Total	Abdomen thor wall	Total
1	4 6	11	—	—	0 12	0 06	2 9	5 8	38 (3?)	87 (33)
2	3 6	10	7 3	8 7	0 14	0 07	1 6	3 8	41 (40)	87 (36)
3	7 0	10	5 5	11	0 09	0 07	2.2	4 0	45 (29)	62 (34)
4	7 0	11	7 5	9 6	0 07	0 05	3 7	7 0	34 (39)	50 (46)
5	6 8	12	6	8 3	0 08	0 06	3 6	7 1	45 (23)	81 (28)
6	3 3	9 4	2 1	9 4	0 13	0 08	0 9	4 1	22 (25)	45 (33)
7	3 5	8 4	2 4	5 9	0 15	0 10	0 5	3 9	53 (16)	82 (34)
8	7 6	14	7	11	0 13	0 08	2 0	3 9	87 (26)	151 (28)
Range	3 3–7 6	8 4–14	2 1–7.5	5.9–11	0 07–0 15	0 05–0 10	0 5–3 7	3 8–7 1	22–87	45–151
9*	10	22	11	21	0 10	0 05	6 5	9 0	61 (29)	133 (19)
10	2 7	10	5 4	—	0.20	0 07	1 0	5 8	19 (32)	72 (49)
11	5 3	14	—	—	0 10	0 06	3.2	10 6	26 (58)	58 (64)
12	4 1	11	11	12	0 13	0 06	1 0	5 6	17 (19)	59 (33)
13	? 7	16	—	—	0 23	0 04	1 6	11 6	15 (33)	91 (65)
14	6 2	17	14	18	0 09	0 05	4 9	14 2	29 (40)	80 (53)
15	4 6	13	8 3	11	0 12	0 11	2 2	7 9	51 (?9)	114 (46)
16	12	17	12	17	0 06	0 05	5 6	9 6	100 (33)	129 (35)
17A	8 4	15	9 1	13	0 08	0 04	1.2	3 3	55 (22)	89 (29)
17B	5 9	10	9 1	14	0 06	0 035	1 7	3 8	60 (25)	103 (30)
18	4 4	10	6 4	14	0 15	0 05	1 6	4 7	42 (39)	96 (37)
Range	2 7–12	10–2?	5 4–14	11–21	0 06–0.23	0 035–0 11	1 0–6 5	3 3–14.2	15–100	58–133

Subjects seated except 10 and 13 who were supine
Fluctuation is twice the ampl tude.
Figures in brackets are inspiratory resistive wo k in percentage of inspiratory work.
T d. = trans-diaphragmatic (from stomach to oesophagus)
With girdle see text.

The final phase of the volume pressure relationship of a slow excessive ex piration (Fig 6) should be the compliance of thorax diaphragm and lungs combined This value determined in the sitting posture except in subjects 8 and 9 who were supine was 0 11 0 12 0 17 0 21 0 07 and 0 11 litre/cm water in subjects 1 4 5 6 8 and 9 respectively

Table III Difference in mechanics of abdomen thoracic wall between supine and sitting position

Subject	In intra-gastric pressure fluctuation (cm H O)	In compliance (l/cm H_2O)	In resistance (cm H_2O/l/sec)	In respiratory work (l cm H O/min)
2	−0 [illegible]	0 01	−0 [illegible]	1
3	−2 0	0 0[illegible]	−0 1	− 7
[illegible]	−2 7	0 09	−0 1	−1[illegible]
8	− 3 3	0 0[illegible]	−0 8	−[illegible]1
9	−4 4	0 02	−2 8	−4[illegible]
14	−1 3	0 0[illegible]	−1 9	—
1[illegible]	−[illegible]	0 03	3 1	−[illegible]4
Mean	−2 8	0 0[illegible]	−1 3	23

Addition to respective value in Table II yields supine position value

Table IV Characteristics of abdomen and thoracic wall

Subject and posture	Compliance (l/cm H O)		Resistance (cm H O/l/sec)		Abdominal fraction of ventilation
	Abdomen	Thorax	Abdomen	Thorax	
4 sitting	0 0[illegible]	0 02	[illegible]2	13	0 93
5 supine	0 12	0 0[illegible]	4	23	0 8[illegible]
9 supine	0 14	0 0[illegible]	2 2		0 [illegible]
10 supine	0 13	0 07	1 3	4 3	0 91
12 sitting	0 11	0 02	1 [illegible]	3 0	0 9[illegible]
11 supine	0 11	0 07	[illegible] 6	[illegible]	0 [illegible]3
17 A sitting	0 07	0 01	2 3	2 5	0 [illegible]
18 sitting	0 10	0 0[illegible]	2[illegible]	4 2	0 [illegible]1
Range	0 0[illegible] 0 14	0 01 0 07	1 3 [illegible]	2 [illegible] 23	0 [illegible]1–0 98

Resistance 0 03 cm H_2O/l sec lower during abdominal than during natural breathing

Ten of the compliance and resistance figures in Table II (studies 1 2, 3 6 7 10 15 17 A 17 B 18) were compared with values obtained during artificial ventilation The volume deflections were then recorded against the mouthpiece pressure (Fig 7) and sometimes the oesophageal pressure (4 subjects) With the exception of one compliance in an oesophageal pressure study and one resistance in a mouthpiece pressure study the values relevant to artificial ventilation were somewhat higher both for compliance and for resistance than those found during spontaneous breathing The mean difference in total compliance was 0 027 litre/cm water range 0 010—0 062) and in total resistance 1 3 cm water/litre/sec (range — 3 6 —4 4)

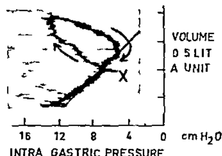


Fig 6 Curve from breath with slow inspiration starting at X followed by slow excessive expiration The straight line drawn at end of expiration shall represent compliance of thoracic wall diaphragm and lungs together Subject 5

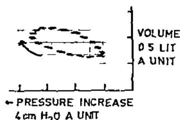


Fig 7 Tidal volume and mouth piece pressure during an artificial ventilation breath Inspiration upwards Subject 17 (B)

Discussion

It is generally agreed that the respiratory pressure fluctuations within the stomach are representative of those in the abdomen (DUOMARCO and RIMINI 1947 CAMPBELL and GREEN 1953 AGOSTONI and RAHN 1960 PETIT *et al* 1960) The ordinary form of the tidal volume gastric pressure loop indicates an inspiratory increase and an expiratory decrease in pressure and conforms with most of the earlier abdominal pressure studies (see DUOMARCO and RIMINI 1947) Such a pressure variation is consonant with an inspiratory contraction of the diaphragm and with passive expiration brought about by gradual relaxation of that muscle without contraction of expiratory muscles (CAMPBELL and GREEN 1953 CAMPBELL 1958 PETIT *et al* 1960) In the curves which showed a reverse extra loop at the end of expiration and at the beginning of inspiration the end expiratory pressure changes are apparently caused by expiratory contraction of abdominal wall muscles This assumption is supported in this study by the fact that they were more frequent and more pronounced during increased breathing The volume pressure loops recorded were of about the same shape as those plotted by AGOSTONI (1961) in 2 subjects

The intra gastric pressure fluctuation during the respiratory cycle was between 2 7 and 12 cm of water — a range approximately corresponding to that reported by CAMPBELL and GREEN (1953) On contraction of the diaphragm the pressure decreases in the thorax but increases in the abdomen If the respiratory muscles are relaxed following expiration the amplitude of the difference between thoracic and abdominal pressures during a breath should reflect the tension developed by the diaphragm The trans diaphragmatic pressure fluctu

ations in Table II suggest the degree of active tension of the diaphragm at the end of inspiration

For calculation of compliance, resistance and respiratory work of abdomen and thoracic wall it is presumed that expiration is passive with no active contraction of abdominal muscles This fits with the regular oval form of the intra gastric pressure tidal volume diagram It has been demonstrated by means of electromyography (CAMPBELL 1958) that the abdominal muscles are little active during ordinary resting breathing The relatively good agreement between values reported in Table II and those during artificial ventilation in ten subjects indicates also that intra gastric pressure may be utilized during resting breathing to calculate the mechanical characteristics of the abdomen and thoracic wall In spite of the general agreement between the results of the two methods there was however in most cases a certain difference in one direction The compliance during active relaxation and artificial ventilation was higher than that recorded during natural breathing when the subject did not cooperate actively This accords with the findings of NIMS, CONNER and COMROE (1955) i e that compliance of chest and lungs is higher during voluntary relaxation than during anesthesia The compliance values from the intra gastric pressure tidal volume loops fall approximately within the range reported to occur at anesthesia or at *small* respiratory level displacements (NIMS *et al* 1955 HOWELL and PACKETT 1957 BROMAGE 1958 DAHLSTROM 1959) but appears somewhat lower than those recorded by other techniques applied to comparable subjects (NIMS *et al* 1955 HEAF and PRIME 1956 NAIMARK and CHERNIACK 1960 MEAD 1960) The total resistance values of the subjects without active pathological conditions are similar to those reported by MEAD (1960) The total work of breathing has previously apparently been studied only in connection with artificial ventilation and intentional relaxation in 3 subjects (OTIS *et al* 1950) and was in about the same range as those given in Table II

There was no clear difference in the mechanics of abdomen and thoracic wall between the normal subjects and the patients studied The compliance of the two subjects with costal fractures was not markedly low This may be because the compliance of the thoracic wall during rest is much lower than that of the abdomen (Table IV) and therefore does not greatly affect the total compliance value

The postural changes in compliance and resistance (Table III) may be attributable to the abdominal wall distension (DUOMARCO and RIMINI 1947) and abdominal muscle tone increase in the erect position (CAMPBELL 1958) The change in compliance was similar to that observed by NAIMARK and CHERNIACK (1960) with another method Hydrostatic forces should tend to increase the intra gastric respiratory pressure fluctuation in supine subjects Since those forces are not involved in deformation of the abdominal wall the actual compliance of the abdominal and thoracic walls may in supine subjects be even higher than that calculated here

The subject appeared to breathe only by means of moving his diaphragm and abdomen when instructed to do so If this breathing was nevertheless partly thoracic (WADE 1954) the abdominal compliance and the abdominal fraction of ventilation during natural breathing will have been overestimated and abdominal resistance underestimated (Table IV) The time constants (compliance × resistance) for natural and abdominal breathing did not differ markedly, and a phase lag between abdomen and thorax was considered to be of little significance in calculations of thoracic characteristics

The gastric curve for slow excessive expiration was utilized to obtain a value for compliance of thorax diaphragm and lungs combined (C_w) which should be related to compliance of thorax and diaphragm (C_{td}) and to compliance of lungs (C_l) according to $1/C_w = 1/C_{td} + 1/C_l$ The compliance of thorax and diaphragm derived be means of this equation was 0 24 litre/cm water in subject 1 0 32 liter/cm water in subject 5 and 0 09 litre/cm water in subject 8 If the expiratory force of the intercostal muscles is greater than that of the abdominal muscles the intra gastric pressure change will be lower than a true mean expiratory pressure and the estimated compliance of thorax diaphragm and lungs will be falsely high That may be the reason why this compliance value is higher than lung compliance in subjects 1 6 and 9 The compliance of the thorax in subjects 4 and 5 (Table IV) may be subtracted from the combined compliance of the diaphragm and thorax 0 24 and 0 32 litre/cm water to obtain apperant compliance values for the diaphragm 0 22 and 0 31 litre/cm water

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concomitant appearance of histamine and Slow Reacting Substance (SRS)[1] suggested that these two agents came from the same source the mast cells (CHAKRAVARTY, HOGBERG and UVNAS 1959 CHAKRAVARTY and UVNAS 1960) A quantitative correlation was observed between the density of mast cell population in guinea pig tissues the release of histamine and the appearance of SRS (BOREUS and CHAKRAVARTY 1960) A SRS was also observed to occur when isolated rat mast cells were exposed to compound 48/80 (UVNAS and THON 1959) The SRS s in question did not appear in significant amounts in extracts or perfusates of normal tissues or mast cells They were formed in connection with the histamine release

Two enzymes, phosphatidase A and chymotrypsin have been reported able to degranulate mast cells and to release histamine from isolated rat mast cells (HOGBERG and UVNAS 1957 1960 MORAN, UVNAS and WESTERHOLM 1962 KELLER 1961) These two enzymes seem to trigger the same energy requiring release process as is activated by *e g* compound 48/80 and antigens (DIAMANT and UVNAS 1961, UVNAS and ANTONSSON 1962) Similar enzymes seem to occur in mast cells (FREDHOLM *et al* 1962 to be published, BENDITT and ARASE 1959) Provided that such enzymes were activated during the histamine release process split products of phosphatides and peptides should appear This paper will be concerned with the separation as well as with certain characteristics of smooth muscle stimulating principles that occur on histamine release in cats guinea pigs and rats

Methods

SRS from Cat Paw

The technique for perfusion of cat paws has been described previously (HOGBERG TUFVESSON and UVNAS 1956) In the present experiments the perfusion fluid consisted of a buffered solution at pH 7 0 and containing 1 5 10^{-1} M NaCl 2 7 10^{-3} M KCl 9 10^{-4} M $CaCl_2$ (anhydrous) and 10 percent 6 7 10^{-2} M Sörensen phosphate buffer The four paws were perfused simulteaneously but separately Following an initial perfusion for 30 min to clear the paws of blood 50 µg 48/80 was given intra arterially The perfusion was continued for 90 min the perfusate being collected in ice chilled glass tubes Following a few minutes centrifugation (at 0 C) for removal of cells and other coarse particles and brief heating to the boiling point the combined material was filtered and lyophilized The dried material was washed with acetone 3 × 50 ml dissolved in a few ml water acidified to about pH 3 with N HCl and mixed with an equal amount of ethanol In the cold room (5 C) the material was shaken twice with 5 volumes of ethyl ether The two ether layers were combined filtered and allowed to pass through a silicic acid column (4 g silicic acid over glass wool in a column 1 cm in diameter)

Provided the ethanol content of the ether did not exceed 5 per cent and the flow rate was below 4 ml/min the SRS was totally retained by the column After washing with ether elution was started with ether chloroform (1 1) and continued with chloroform and chloroform methanol the methanol content being stepwise increased

[1] SRS is defined as (a) principle(s) causing a slow contraction of the atropinized and mepyraminized guinea pig ileum without tachyphylaxis requiring several minutes for relaxation

For re-chromatography of SRS on silicic acid the chloroform methanol (1 1) was concentrated *in vacuo* + 60 C to near dryness dissolved in a few ml methanol and then diluted with chloroform to 10 per cent methanol before being placed on a new column After chloroform methanol (9 1) the SRS activity was eluted with chloroform methanol (1 1)

For biological assay the eluates were evaporated *in vacuo* to dryness and then dissolved in a small volume of methanol and then either tested directly or diluted with physiological saline buffered to pH 7 4 with isotonic phosphate buffer Care was taken not to poison the assay organ with methanol Methanol in the bath fluid exceeding 0 5 % disturbed the assay

SRS from Guinea Pig Lung

Guinea pigs were sensitized with crystalline egg albumin 100 mg subcutaneously and 100 mg intraperitoneally After 4—10 weeks the lungs were removed from the sensitized animals and chopped with scissors The material was washed twice in a slightly modified Tyrode solution (Sorensen phosphate buffer instead of bicarbonate) and then incubated in the same solution with antigen 1 mg/ml for 20 min at 37 C and pH 7 (20 ml/lung) The incubation fluid was boiled for 3 min and lyophilized The incubated lung tissue was suspended in buffered Tyrode solution (5 ml/lung) and boiled for 3 min Ethanol was added to a concentration of 80 per cent After 30 min extraction at room temperature the fluid was filtered off concentrated *in vacuo* until foaming occurred and then lyophilized

The lyophilized material was washed repeatedly with acetone (in most experiments) extracted and chromatographed on silicic acid as described for material from cat paws The dried SRS containing eluate was dissolved in 0 5 M NaOH and shaken in the cold room (5 C) with ether to remove inert material After removal of the ether and evaporation of the ether rest the alkaline water solution was cooled in ice water and carefully acidified with HCl to a pH around 3 and shaken twice with ether containing 5 % ethanol The SRS-containing ether was filtered and allowed to pass through a second silicic acid column Elution was performed as described for cat paw SRS

For perfusion lungs were removed from guinea pigs killed by a blow on the head The lungs were perfused with Tyrode solution via a cannula inserted into the right ventricle The perfusion fluid was lyophilized the dry residue washed with acetone and extracted with 80 per cent ethanol After evaporation of the ethanol and subsequent lyophilization the SRS was isolated and purified in the same manner as described for cat paw SRS

SRS from Rat Mast Cells

Peritoneal rat mast cells were isolated *ad modum* Uvnas and Thon (UVNAS and THON 1959) The mast cells were exposed to 48/80 as described by those authors and SRS was extracted and purified as in the case of cat paw SRS

Perfusion of mast cells was performed as follows The lower part of a small burette (7 mm inner diameter) was filled with glass wool to support a 3 cm high column of Whatman cellulose powder Coarse grade (W & R Balston Ltd) A suspension of mast cells was carefully poured into the burette When the cells had settled they were covered with a thin layer (about 1 mm) of cellulose powder All mast cells were retained by the cellulose powder even when this was perfused at a rather high rate (5 ml/min) The mast cells were perfused at a constant rate (5 ml/min) and compound 48/80 was added to the perfusion fluid by careful injection into the burette just above the surface of the cellulose powder column For composition of perfusion fluid see UVNAS and THON (1959) The perfusate was tested directly for histamine and SRS

Assay Technique

Histamine was assayed on atropinized (1 5 10^{-6} M) guinea pig ileum SRS was as sayed on atropinized (1 5 10^{-6} M) and mepyraminized (1 25—2 5 10^{-7} M) guinea pig ileum or on atropinized 1 5 10^{-6} M) rabbit duodenum SRS was assayed against a standard preparation of cat paw SRS The activity was recorded in arbitrary units For further details about the biological assay technique see p 103

Paper Chromatography

Paper chromatography was performed on Whatman paper no 1 (according to HANES and ISHERWOOD 1949) in n propanol ammonia water (6 3 1) descending at 5 C for 16 hours on silicic acid impregnated paper in di isobutylketone acetic acid water (40 20 3) ascending at 5 C for 16—20 hours (MARINETTI 1962), and on formalin treated paper in n butanol acetic acid water (supernatant phase of 4 1 5) descending at 5 C for 21 hours (HORHAMMER WAGNER and RICHTER 1959) If possible chromatograms were run parallel for chemical and biological analysis For biological assay the papers were extracted in methanol (60 C) and the extracts tested on the guinea pig ileum

Spot tests

Aldehyde (MARINETTI 1962 E MERCK AG)
Carboxyl groups (FEIGL 1954)
Acyl ester linkages (WHITTAKER and WIJESUNDERA 1952)
Choline (MARINETTI 1962)
Phosphorus (SMITH 1960)
Phosphatides (MARINETTI 1962)
Ninhydrin (SMITH 1960)
Ehrlich (SMITH 1960)
Orcinol (KLEVSTRAND and NORDAL 1950)
Sugar (Anilin-difenylamin) (SMITH 1960)

The radioactive spots on the paper chromatograms were detected by means of an automatic chromatogram scanner (thin mica window G-M tube) or by pressing the chromatograms against Kodak no screen X ray film The exposure time varied between one and two weeks

Materials

Compound 48/80 was synthetized by the method of Baltzly *et al* (BALTZLY *et al* 1949)
Phosphatidase A was obtained from bee venom by chromatography on Amberlite IRC 50 (XE 64)
Silicic acid (Unisil 100—200 mesh) was kindly supplied by Dr Evan C Horning Houston Texas USA It was activated by heating to 115 C for one hour
Ether anhydrous peroxide free (Mallinckrodt)
Acetone (analytical grade)
Chloroform (analytical grade) Freshly distilled

Results

Regardless of releaser tissue or technique used, release of histamine was invariably accompanied by the appearance of SRS On chromatography on silicic acid the spasmogenic material especially material from incubates could be separated *into several components Due to different sensitivity to the* eluted

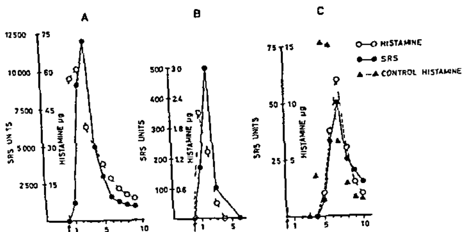


Fig 1 Histamine and SRS in perfusates from cat paw (A) guinea pig lung (B) and isolated rat mast cells (C) Releasing agent in A compound 48/80 50 µg/paw in B antigen = egg albumin 10 mg/lung in C compound 48/80 1 µg/ml
Perfusion A 9 × 10 min B 6 × 3 min C 10 × 6 sec.

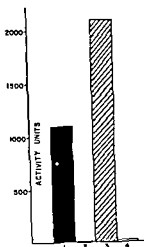


Fig 2 Separation by silicic acid chromatography of spasmogenic principles in perfusate from cat paw exposed to compound 48 80
1 Chloroform *2* Chloroform methanol 9 + 1 *3* Chloroform methanol 1 + 1 *4* Methanol

principles of the two assay organs used (guinea pig ileum and rabbit duodenum) the activity patterns of the chromatograms varied according to the assay technique (Fig 4) When not otherwise stated the results described below were obtained from assays on guinea pig ileum The incubation time was another influencing factor since several of the spasmogenic principles formed were rapidly inactivated Inactivating factors occurred in the mast cells themselves (Fig 6) in serum (Fig 8) in the lung and in various other organs investigated (to be published)

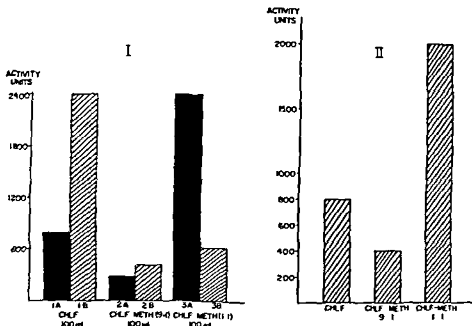


Fig 3 Separation by silicic acid chromatography of spasmogenic principles from sensitized guinea pig lung (I) and isolated rat mast cells (II)
I Releaser egg albumin. A incubation fluid B lung tissue
II Releaser compound 48/80

Separation of spasmogenic principles by silicic acid chromatography

Fig 1 illustrates the appearance of histamine and SRS from *perfusates* of cat paw (A) guinea pig lung (B) and isolated rat mast cells (C) On chromatography on silicic acid the spasmogenic activity was found mainly (guinea pig lung and isolated rat mast cells) in the chloroform methanol (1 1) fraction The cat paw in addition delivered spasmogenic material in the chloroform fraction (Fig 2) The spasmogenic activity in this fraction varied with the condition of the paws The longer these were stored in the refrigerator before perfusion the more activity was found in the chloroform eluate and the less in the chloroform methanol (1 1)

The SRS *material from incubates of guinea pig lungs and mast cells* showed a *more composed chromatogram Considerable spasmogenic* activity was found both in the chloroform and in the chloroform methanol (1 1) eluates A slight spasmogenic activity was observed also in the chloroform methanol (9 1) eluate (Fig 3) but on rechromatography this activity parted into two fractions one appearing in chloroform the other in chloroform methanol (1 1)

From Fig 3 also emerges the distribution of active material between tissue and incubation fluid The more lipophilic material (eluted with chloroform)

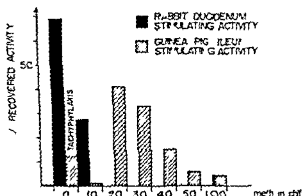


Fig 4 Assays on guinea pig ileum and rabbit duodenum of spasmogenic principles separated on silicic acid column. Note that rabbit duodenum stimulating material is eluated in less polar fractions whereas the guinea pig ileum stimulating material appears in the more polar eluates

was mostly retained by the tissue while most of the material eluted with chloroform methanol (1:1) appeared in the incubation fluid This distribution may explain why chromatograms of perfusates exhibited spasmogenic activity only or mainly in the chloroform methanol (1:1) eluates

Spasmogenic properties of the separated principles

The fractions separated by silicic acid chromatography differed considerably in their spasmogenic properties

The principle(s) in the chloroform eluate elicited a rather rapid transient contraction of the guinea pig ileum The contraction therefore was not a typical SRS effect Tachyphylaxis occurred after a few contractions It was observed that the development of tachyphylaxis was prevented by interposed doses of unpurified SRS (perfusate from cat paw) Biological assay of chloroform eluates was therefore possible on the guinea pig ileum provided that the two principles were added to the test bath alternately

Unsaturated fatty acids are known to elicit a rapid transient contraction of the guinea pig ileum and to exhibit tachyphylaxis on that test organ (VOGT 1957 ÄNGGÅRD and BERGSTRÖM 1962) They are eluted from silicic acid columns with chloroform (BORGSTRÖM 1952 VOGT 1960) They also stimulate the rabbit duodenum as did the principle(s) in our chloroform eluates (see Fig 4) The spasmogenic activity could therefore well be due to the presence of unsaturated fatty acids

The principle(s) eluted with chloroform methanol (1:1) elicited a typical SRS contraction The contraction was slow and required about 3 min for relaxation The spasmogenic activity was more or less confined to the guinea pig ileum With purified material a concentration in the test bath of 0 001 $\mu g/ml$ sufficed to elicit a contraction of the guinea pig ileum while to stimulate the rabbit duodenum doses 400—600 times as high were required No tachyphylaxis

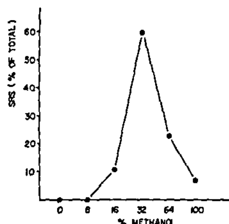


Fig 5 Rechromatography on silicic acid of SRS from cat paw perfusate Elution with chloroform with increasing percentage of methanol Eluate volumes 100 ml

was observed The contractile actions of histamine and bradykinin on guinea pig ileum were potentiated No action was observed on the rat small intestine, the oestrogen treated rat uterus and the non pregnant guinea pig uterus with doses 100—500 times the threshold dose on the guinea pig ileum

Assay on the rabbit duodenum yielded a quite different spasmogenic activity pattern of the chromatographed material (Fig 4) The activity was mainly confined to the chloroform fraction Eluates containing 20 per cent methanol or more showed only traces of activity

Properties of SRS (the chloroform methanol (1 1) fraction)

Our interest was focused on the principle in the chloroform methanol (1 1) fraction since the contraction on the guinea pig ileum elicited by this principle was similar in type to that caused by the original perfusates This contraction was typical for a SRS

Rechromatography of SRS on silicic acid column gave the elution curve seen in Fig 5 Such rechromatographed material was subjected to paper chromatography (n propanol ammonia water) For technical reasons the exact extension of the area of biological activity was difficult to establish since in order to obtain sufficient material for reliable assay the chromatography paper had to be cut into rather broad strips Most of the active material could be extracted from an area between Rf 0 7—0 8 The Rf values varied somewhat due to the pretreatment of the paper and the temperature

When SRS material from guinea pig lung and isolated mast cells was chromatographed against SRS from cat paw as reference material the three SRS s yielded identical Rf values (Fig 7)

Within the area of SRS activity were obtained positive staining reactions for phosphatides phosphorous choline aldehyde carboxyl groups and acyl ester linkages When SRS was prepared from cats guinea pigs or rats given 0 5 mC

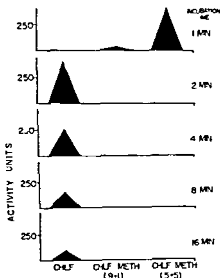


Fig 6 Silicic acid chromatography of spasmogenic principles from isolated rat mast cells exposed to compound 48/80 1 μg/ml Number of mast cells approx. 5 10^6 Temperature 37 C. Note the influence of incubation time on the presence of spasmogenic activity in the eluates.

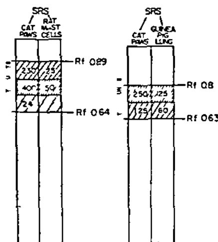


Fig 7 Paper chromatogram (n propanol ammonia water) of SRS-material from cat, guinea pig and rat twice chromatographed on silicic acid Note close similarity of Rf values from the three species

^{32}P i m /kg 18 hours before perfusion or incubation the paper chromatogram showed a radioactive spot within the area of biological activity The ninhydrin reaction sugar and orcinol tests were negative in this area

When SRS material (purified on silicic acid columns) was subjected to chromatography on silicic acid impregnated paper the radioactive spot (yielding positive staining reactions for phosphatides aldehyde and choline) showed an Rf value corresponding to phosphatidyl choline (egg lecithine) and phosphatidal choline Identical results were obtained with chromatography on for

Table I Identification of SRS bearing phosphatides with phosphatidyl (and phosphatidal) choline

Chromatography on silicic acid impregnated paper according to MARINETTI 1962 Rf values

	SRS-fraction	SRS fraction + phosphatidase A	*Lecithin*[1]	*Lysolecithin*
Rodamin 6 G	0 59	–	–	–
DNPH[2]	0 60	–	–	–
Choline	0 62	–	–	–
DNPH	0 57	–	–	–
Choline	0 56	–	0 56	–
DNPH	0 63	–	0 65	–
Choline	0 43	0.20	0 45	0 21
Autoradiogram P	0 64	0 36	–	–
Choline	0 65	0 35	–	–
Autoradiogram P[3]	0 46	0.26	–	–
Choline	0 46	0 26	0 46	0 27
Chromatography on formalin treated paper according to HORHAMMER et al 1959 Rf values				
Autoradiogram P	0 82	0 67	–	–
Phosphate reagent	–	–	0 81	0 67

The Rf values of SRS vary between different experiments due to difficulties in preparing silicic acid impregnated papers of equal qualities

[1] Egg lecithin contains some phosphatidal choline

[2] 2 4 dinitrophenylhydrazine

malin treated paper After incubation of SRS material with phosphatidase A chromatography of the incubated material on silicic acid impregnated paper revealed a phosphorus and choline positive spot corresponding to lysolecithine No other phosphorus and choline positive spot was to be seen (Table I)

The solubility properties, the behaviour on silicic acid column and on n propanol ammonia water paper suggested to us that SRS could be a choline containing phosphatide However, further analysis contradicted this assumption The phosphorus content – below 2 per cent — was too low for a phosphatide and the peak of radioactivity usually did not completely coincide with the area of maximal SRS activity Further the SRS activity was resistant to the action of phosphatidase A — as it was to phosphatidases B, C and D

Since the above results indicated that the SRS was not a phosphatide but coupled to or merely mingled with such material silicic acid chromatographed material was thoroughly washed with chloroform to remove phosphatides The SRS activity was found to be retained in the chloroform insoluble material

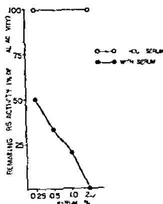


Fig 8 Inactivation of SRS (from cat paw chromatographed on silicic acid column) by guinea pig serum Temperature 37 C Incubation time 60 min

and the SRS now became easily soluble in water made slightly alkaline (from pH 7 8) When subjected to paper chromatography (n propanol ammonia water or n butanol acetic acid water) several ninhydrin spots now turned up two of which gave yellow and one violet staining with Ehrlich s reagent When the alkaline water solution was shaken with n butanol the SRS material was found in the butanol layer On electrophoresis (according to CHARLWOOD and GORDON 1958) of the lyophilized butanol the SRS material was obtained within a rather narrow sector Examination of this material continues

Since SRS activity was found to be resistant to trypsin chymotrypsin carboxypeptidase and leucine aminopeptidase SRS is probably not a polypeptide

The nature of the enzymatic inactivation of SRS by degranulated mast cells (Fig 6) serum (Fig 8) and various tissue extracts is under investigation

Discussion

A slow reacting substance (SRS) is known to accompany anaphylactic histamine release in guinea pig lung (KELLAWAY and TRETHEWIE 1940 BROCKLEHURST 1960 CHAKRAVARTY and UVNAS 1960) and histamine release in cat paw induced by compound 48/80 (CHAKRAVARTY HOGBERG and UVNAS 1959) and by extracts from *Cyanea* and *Ascaris* (UVNAS and THON unpublished observations) It also occurs in isolated rat mast cells exposed to compound 48/80 (UVNAS and THON 1959) and to antigen

As shown in the present investigation the original SRS material from cat paw guinea pig lung and isolated rat mast cells can be separated into several active fractions which differ markedly in their chemical and biological behavior One fraction contains a principle yielding a typical SRS contraction on the guinea pig ileum and another fraction could contain unsaturated fatty acids

Both biologically and chemically the SRS — whether it comes from cat paw guinea pig lung, or isolated rat mast cells — shows the same characteristics There is therefore no experimental evidence to justify the differentiation of the SRS s *formed in connection with histamine release due to anaphylactic re actions* — designed as SRS A by BROCKLEHURST — and to compound 48/80 (and other polymer releasers)

The spasmogenic action of SRS is very specific, guinea pig ileum being by far the most sensitive smooth muscle preparation of those tested by us The SRS s studied by BROCKLEHURST and CHAKRAVARTY and others were reported to stimulate the rabbit small intestine fowl rectal caecum and rat colon These stimulating effects were probably due to the presence of other active principles in their preparations Prior to silicic acid chromatography our SRS materials from both cat paw and guinea pig lung exhibited the same wide range of spasmogenic activity but on purification of SRS the actions on other smooth muscle organs than guinea pig ileum progressively declined

The chromatographic analysis showed that the SRS material was mingled with phosphatidyl choline and phosphatidal choline It remains to be elucidated whether this coupling ' of SRS to phosphatides is an artefact occurring during the preparation or if it reflects its origin from lipid structures

It has recently been claimed that SRS is a neuraminic acid derivative (SMITH 1962) Our chloroform washed SRS did not show any positive staining reaction for neuraminic acid (Bial s orcinol reaction) Our SRS is far more active on the guinea pig ileum than neuraminic acid and its acetylated derivative Our *observations therefore do not support Smith s statement*

Our observations do not indicate that SRS is a polypeptide Whether or not it is identical with any previously known substance or is a new one remains to be elucidated Its specific action on guinea pig ileum favours the idea that it is not identical with any structurally identified substance

The functional significance of the SRS is so far unrevealed It may play a role in the release process in the mast cells but its mechanism of action is com pletely unknown As mentioned, the serum of e g guinea pig contains an SRS inactivating factor Of great interest is our observation that the inactivating power of guinea pig serum is considerably reduced in sensitized animals (to be published)

The degranulation of mast cells and the release of histamine and 5HT depend on an energy requiring enzyme mechanism (HOGBERG and UVNÄS 1960 DIAMANT and UVNÄS 1961 UVNÄS and THON 1961 MORAN UVNÄS and WES-TERHOLM 1962) The details of this enzyme mechanism are unknown but it has been suggested that the initiating process in the mast cell membrane entails the activation of phosphatidase A or a related lipolytic enzyme (HOGBERG and UVNÄS 1960) Acid lipids capable of activating smooth muscle have been found to accompany histamine release not only from tissues but also from isolated mast cells They may be unsaturated fatty acids formed by splitting of phos

phatides in the mast cells Whether or not the occurrence of the spasmogenic lipids reflects the activation of a phosphatidase A in the histamine release process remains to be clarified

The hypothesis of phosphatidase A activation as a link in the histamine release process requires the existence of such an enzyme in the mast cells Observations made with the Cartesian diver technique indicate that the rat mast cells harbour a factor that splits lecithin but not lysolecithin (FREDHOLM *et al* 1962 to be published) These observations are suggestive of a phosphatidase A action localized to the mast cell

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From the Department of Physiological Chemistry University of Lund Sweden

The Digestion and Absorption of Maltose and Trehalose by the Intact Rat

By

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Abstract

Dahlqvist A and D L Thomson *The digestion and absorption of maltose and trehalose by the intact rat* Acta physiol scand 1963 *59* 111—125 — The rates and sites of absorption of equal amounts of maltose and trehalose were studied and compared with those of their component monosaccharide glucose Using polyethylene glycol as a water soluble nonabsorbable marker the transits through the gastrointestinal tract and the absorption indices for the sugars in the various segments were calculated The rates and sites of absorption of the disaccharides were also compared with the amounts and localizations of the corresponding disaccharidases in the intestinal wall The rates and loci of maltose and glucose absorption were the same The rate of the intracellular hydrolysis of maltose was not rate limiting for its absorption The rate of trehalose absorption during the first 2 hr after administration was not significantly different from that of glucose During the later phase of trehalose absorption when the remaining trehalose was in the lower small intestine and colon this sugar was absorbed considerably more slowly This suggests that the relative lack of trehalase in the lower small intestinal mucosa limited the rate of absorption of trehalose at this site A considerable amount of trehalose was absorbed from the lower third of the small intestine and the colon in contrast to glucose and maltose

Maltose is the principal end product of the digestion of utilizable dietary polysaccharides by salivary and pancreatic amylase both in man and the rat It is thus a major dietary constituent Trehalose is a rare disaccharide present in fungi and insects (Pigman 1957) Like maltose it is composed of two glucose molecules It is hydrolyzed more slowly by extracts of the intestinal mucosa

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than maltose and trehalase also has a different distribution in the small intestine than maltase (DAHLQVIST 1961 a 1962) Information about the digestion and absorption of trehalose may therefore contribute to our knowledge of the physiology of the intestinal disaccharidases

In recent studies on the digestion and absorption of disaccharides in the human using an intubation technique with a non absorbable marker (BORGSTRÖM *et al* 1957 DAHLQVIST and BORGSTROM 1961), the disaccharidases were found to be localized in the mucosal cells rather than secreted into the intestinal juice Because of their intracellular localization, quantitative information about the intestinal disaccharidases cannot be obtained using intubation techniques of this kind Neither can the total rate of absorption of the sugars be measured, since the intestinal contents cannot be recovered *in toto*

Data concerning the rate of sugar hydrolysis and absorption the site of the absorption and the amount and localization of the intestinal hydrolytic enzymes can be obtained by using intact rats for absorption experiments When a water soluble and non absorbable reference substance is used at the same time the transit through the gastrointestinal tract and the absorption indices in the different parts of the gut can be measured Using polyethylene glycol as the nonabsorbable reference substance we have previously studied the digestion and absorption of sucrose in the intact rat in this way (DAHLQVIST and THOMSON 1963) We are now reporting a study of the digestion and absorption of maltose and trehalose in the rat performed with the same technique The data on the absorption of these two disaccharides has been compared with that on the absorption of a corresponding amount of glucose

Materials and Methods

Animals Albino rats of both sexes of the Sprague Dawley strain were used The weight of the rats varied between 150 and 196 g with a mean of 170 g Before the experiment the rats were fasted for 16—20 hours with free access to water

Sugars *Glucose* was obtained from Baker Chemical Co (Phillipsburg N J U S A) *maltose* (4 (α D-glucopyranosyl) D glucose) monohydrate and *trehalose* (1 (α D glucopyranosyl) α D glucopyranoside) dihydrate from Pfanstiehl Chemical Laboratories (Waukegan Ill U S A) All sugars were of analytical grade purity

Expression of the weight of sugars On complete hydrolysis 1 0 g of maltose monohydrate will yield 1 0 g of glucose and 1 0 g of trehalose dihydrate will yield 0 95 g of glucose In order to simplify the calculations the amount of maltose or trehalose found in the analyses has always been expressed as the weight of the glucose formed on hydrolysis

Absorption tests The absorption tests were performed in the same way as that previously described for sucrose and invert sugar (DAHLQVIST and THOMSON 1963) Each rat was given by stomach tube 800 mg of one of the three sugars tested together with 50 mg of polyethylene glycol dissolved to a volume of 4 ml with distilled water At the conclusion of the absorption period the entire gastrointestinal tract was removed and divided as previously described After the disaccharidases had been inactivated by heating homogenates of the stomach upper middle and lower thirds of the small intestine and the large intestine were prepared and diluted to 100 ml The homogenates were stored at minus 20 C until the analyses were performed

Analytical methods The determination of polyethylene glycol and the precipitation of protein were performed as described earlier (DAHLQVIST and THOMSON 1963) The sugars were determined individually using the deproteinized homogenate First the amount of free glucose present was determined with the tris-glucose oxidase reagent of DAHLQVIST (1961 b) which does not react with maltose or trehalose Then the total amount of sugar present was determined with the anthrone method performed essentially as described by SCOTT and MELVIN (1953) but with heating at 100 C for 7 5 min instead of at 90 C for 16 min Glucose was used for the preparation of the standard curve The molar extinction for glucose with this method is the same whether it is present as free glucose or as maltose or trehalose Thus the amount of disaccharide present could be calculated as the amount of sugar found with the anthrone reagent minus the amount of glucose found with the tris glucose oxidase reagent Deproteinized homogenates of the small intestine of fasting rats did not contain anthrone positive material corresponding to more than a few mg of glucose

The amount of sugar excreted into the urine was determined as described previously (DAHLQVIST and THOMSON 1963)

Determination of the rate of hydrolysis of maltose and trehalose in vitro by tissue homogenates The gastrointestinal tract of fasting rats was removed and divided into segments The stomach and the large intestine were cut open and the contents removed Each segment was weighed and then homogenized for 2 min in an Ultra Turrax homogenizer with 4 parts (v/w) of 0 9 per cent sodium chloride The tube was chilled with crushed ice during the homogenization The homogenate was centrifuged at 1 000 × g for 10 min The opalescent supernatant which contains the disaccharidases (BORGSTRÖM and DAHLQVIST 1958) was analyzed for maltase and trehalase activity under the conditions described by DAHLQVIST (1960) using the tris-glucose oxidase reagent (DAHLQVIST 1961 b) for the determination of the degree of hydrolysis One unit of maltase or trehalase liberates 1 mg of glucose from the appropriate substrate in 60 min under the conditions used

Calculation of the results

The terms used are the same as those reported previously (DAHLQVIST and THOMSON 1963) They are modified from those used by REYNELL and SPRAY (1956 a)

Gastric emptying is calculated using the formula

$$\text{Gastric emptying} = \frac{P_m - P}{P_m} \times 100$$

P_m = mg polyethylene glycol administered
P = mg polyethylene glycol recovered from the stomach

Intestinal transit is that percentage of the marker entering a given segment during the time since intubation which has moved on to the next segment during the same time interval

$$\text{Intestinal transit} = \frac{P_d}{P_d + P} \times 100$$

P_d = mg polyethylene glycol recovered from all parts of the intestinal tract distal to the segment under consideration
P = mg polyethylene glycol recovered from the segment of intestine under consideration

Total amount of sugar absorbed is the amount of sugar in mg which has disappeared from the gastrointestinal tract during the absorption period It is calculated by subtracting the total amount of sugar recovered (glucose and maltose or trehalose when the disaccharides were fed) from the amount fed

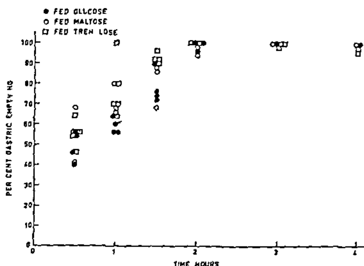


Fig 1 Gastric emptying calculated from the amount of polyethylene glycol recovered from the stomach at different times after the intragastric administration of 800 mg of sugar and 50 mg of polyethylene glycol in 4 ml of water

Rate of sugar absorption is expressed as the number of mg of sugar absorbed per hr as calculated from all values obtained during the first two hours after intubation

Absorption index is the percentage of sugar which has been absorbed from that fraction of the solution fed which is present in the segment under consideration at the time the animal is killed The fraction of the amount of polyethylene glycol fed which is found in the segment is taken to represent the fraction of the original solution present in the segment The absorption index thus provides a measure of the degree of absorption of the sugar

It is calculated from the formula

$$\text{Absorption index} = 100\left(1 - \frac{S}{P}\frac{P_m}{S_m}\right)$$

S = mg of sugar recovered from the relevant segment
P_m = mg of polyethylene glycol administered
P = mg of polyethylene glycol recovered from the relevant segment
S_m = mg of sugar fed

The intestinal transit and absorption indices were only calculated for segments in which at least 10 % (5 mg) of the amount of polyethylene glycol fed was recovered

Statistical calculations The regression coefficients means variances and standard errors were calculated and compared according to BAILEY (1959)

Amount and rate of hydrolysis of the disaccharides in vivo The amount of disaccharide hydrolyzed *in vivo* was calculated using the assumption that all of the disaccharide (maltose or trehalose) which had disappeared from the gastrointestinal tract at a given time had been hydrolyzed during its absorption The amount of sugar hydrolyzed was thus calculated as the sum of the amount of sugar which had disappeared from the gastrointestinal tract plus the amount of free glucose recovered in all of the segments The rate of hydrolysis of the disaccharides *in vivo* (mg/hr) was calculated from the values obtained during the first hr after intubation

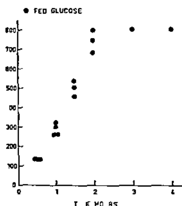


Fig 2

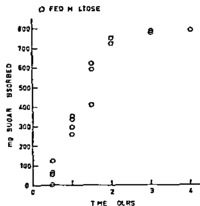


Fig 3

Fig 2 Total amount of sugar absorbed at different times after the intragastric administration of 800 mg of glucose

Fig 3 Total amount of sugar absorbed at different times after the intragastric administration of 800 mg of maltose

Results

Recovery of polyethylene glycol Of the 50 mg of reference substance fed 48 4 ±0 58 mg were recovered (mean ± S E)

Gastric emptying

The percentage of gastric emptying after different time intervals was the same whether the rats were fed glucose or maltose After the feeding of trehalose the gastric emptying seemed to proceed a little more rapidly (Fig 1) In all animals the gastric emptying exceeded 55 % in 1 hr and 95 % in 2 hr

Rate of sugar absorption

The total amounts of the sugars absorbed at varying times after the feeding of 800 mg of glucose, maltose or trehalose are shown in Fig 2 3 and 4

The absorption curves for glucose and maltose were very similar (Fig 2 and 3) The absorption of these sugars proceeded linearly until the absorption was over 90 % complete

The absorption curve for trehalose was obviously different from those of the other two sugars After 2 hr 67 % of the trehalose absorbed and after that time the absorption proceeded more slowly (Fig 4)

The absorption of all three sugars appeared to proceed linearly during the first 2 hr The regression coefficients for this period were calculated with the assumption of a linear absorption The regression coefficients for the glucose maltose and trehalose experiments were 444 457 and 302 respectively The

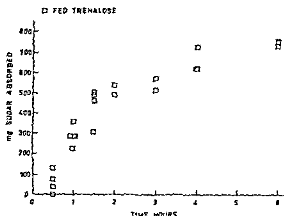


Fig 4 Total amount of sugar absorbed at different times after the intragastric administration of 800 mg of trehalose

equations for the corresponding regression lines were $y = -145 + 444x$, $y = -158 + 457x$ and $y = -48 + 302x$

The regression lines for the absorption of glucose and maltose were nearly identical The line for the absorption of trehalose had a smaller slope but this difference was not found to be statistically significant when the regression coefficients were compared using a modified Student s t test (BAILEY 1959)

Using the values for the first 2 hr the rates of sugar absorption in mg per hr were also calculated For this period glucose was absorbed at the rate of 297 ± 19 mg per hr (mean ± S E) maltose at the rate of 271 ± 35 mg per hr and trehalose at the rate of 235 ± 27 mg per hr These rates were not significantly different when compared in pairs using the Student s t test

Therefore the rate of sugar absorption during the first 2 hrs was not significantly different whether the 800 mg of sugar were fed as glucose maltose or trehalose although the later part of the absorption curve for trehalose clearly differed from those of the other two sugars

Intestinal transit

The transit through the three segments of the small intestine at different times after intubation is seen in Table I During the first three hours the values were similar for all of the three sugars fed The transit decreased progressively from the upper to the lower third of the small intestine at each time interval

After 2 hrs over 95 % of the polyethylene glycol fed had entered the middle third of the small intestine and after 3 hrs over 95 % had entered the lower third (Fig 1 and Table I) When glucose and maltose were fed 98 % of the polyethylene glycol had entered the large intestine after 4 hrs but after the feeding of trehalose half of the polyethylene glycol remained in the lower third of the small intestine even after 6 hrs

Table I Mean intestinal transit of the sugar polyethylene glycol solutions through the small intestine at various times after feeding

Time after intubation (hr)	No of animals	Sugar fed	Small intestine					
			Upper third		Middle third		Lower third	
			Mean	Range	Mean	Range	Mean	Range
0 5	4	Glucose	59	47 74	41	22–70	18	0–71
	4	Maltose	57	53–60	34	14–53	17	0–25
	4	Trehalose	71	62–91	41	35–45	0	—
1 0	4	Glucose	70	61–77	46	31–75	0	—
	4	Maltose	73	66–79	41	28–63	4	0–9
	4	Trehalose	87	72–98	54	30–83	22	0–67
1 5	3	Glucose	91	85–95	66	38–89	1	0–4
	3	Maltose	93	90–95	53	32–70	5	0–11
	4	Trehalose	94	88–97	67	33–91	39	0–71
2.0	3	Glucose	98	—	93	93–94	22	0–65
	2	Maltose	98	—	87	80–95	2	—
	2	Trehalose	100	—	94	92–96	55	51–60
3 0	1	Glucose	100	—	100	—	70	—
	2	Maltose	99	98–100	99	98–100	12	—
	2	Trehalose	99	98–100	95	—	48	45–52
4 0	1	Glucose	100	—	100	—	100	—
	1	Maltose	98	—	100	—	98	—
	2	Trehalose	98	—	98	—	41	37–46
6 0	2	Trehalose	99	98–100	99	98–100	53	29–78

Type of sugar recovered from the various segments

The amounts and types of sugar recovered from the different segments at various times after the feeding of 800 mg of glucose maltose and trehalose are shown in Fig 5 6 7

When the disaccharides (maltose or trehalose) were fed only minute amounts of free glucose were recovered from the stomach at any time indicating that very little hydrolysis occurred at this site (Fig 6 and 7)

When glucose or maltose was fed the amounts of sugar recovered from the lower third of the small intestine and from the large intestine were always small (Fig 5 and 6) After the feeding of maltose up to 50 % of the sugar recovered from the small intestine was free glucose (Fig 6)

When trehalose was fed in contrast considerable amounts of sugar were recovered from the lower third of the small intestine and from the large intestine (Fig 7) As much as 20 % of the total amount of trehalose administered

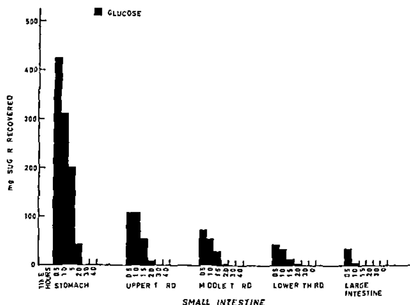


Fig 5 Mean amounts of glucose recovered from different parts of the gastrointestinal tract after the intragastric administration of 800 mg of glucose

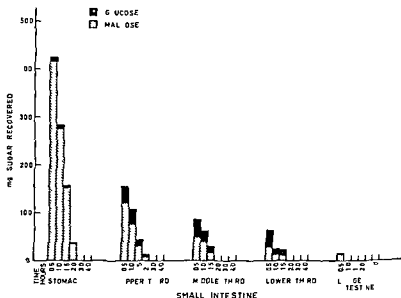


Fig 6 Mean amounts of glucose and maltose recovered from the various segments of the gastrointestinal tract after the intragastric administration of 800 mg of maltose

was recovered from the large intestine The amounts of free glucose recovered after the feeding of trehalose were much smaller than those recovered after the feeding of maltose

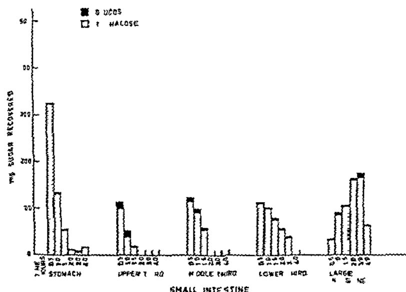


Fig 7 Mean amounts of glucose and trehalose recovered from the various segments of the gastrointestinal tract after the intragastric administration of 800 mg of trehalose

Gastric and intestinal absorption indices

Very little sugar was absorbed in the stomach and in many experiments no gastric absorption at all could be detected (Table II)

Most of the glucose and maltose fed was absorbed in the upper two thirds of the small intestine Absorption was practically complete in samples obtained distally to the middle third of the small intestine after the administration of these sugars except in a few samples recovered from the lower third of the small intestine during the first hour after intubation

After the feeding of trehalose however only about 50 % of the sugar was absorbed in the samples recovered from the large intestine as late as 3 hrs after intubation (Table II)

Completeness of the absorption of maltose and trehalose

In the experiments where the absorption had proceeded for 6 hr less than 2 % of the sugar but over 90 % of the polyethylene glycol was recovered from the gastrointestinal tract This indicates that the absorption proceeded to completion In other experiments the faeces of the rats were collected for 2 days after the feeding of the sugar solutions Less than 1 % of the sugar fed was recovered

Table II Mean absorption indices for glucose maltose and trehalose for the various gastro intestinal tract segments at various time intervals after intubation

Time after intubation (hr)	No of animals	Sugar fed	Stomach		Small intestine						Large intestine	
					Upper third		Middle third		Lower third			
			Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
0.5	4	Glucose	2	0–6	26	13–42	40	26–57	36	–	–	–
	4	Maltose	0	0	13	0–22	42	30–58	22	–	–	–
	4	Trehalose	6	0–21	8	0–26	31	19–44	13	0–31	–	–
1 0	4	Glucose	6	0–14	22	16–29	70	64–77	72	59–97	–	–
	4	Maltose	6	0–13	17	0–28	69	61–79	78	72–89	–	–
	4	Trehalose	7	0–13	25	0–35	47	28–61	43	28–86	20	–
1 5	3	Glucose	4	0–6	8	0–25	85	78–92	94	86–99	–	–
	3	Maltose	2	0–7	0	0	65	22–88	89	72–98	–	–
	4	Trehalose	7	0–15	38	0–87	76	64–91	67	53–81	43	40–51
2 0	3	Glucose	0	0	46	0–94	94	88–100	93	89–96	100	–
	2	Maltose	2	0–4	19	0–38	9[illegible]	94–96	98	–	–	–
	2	Trehalose	–	–	–	–	93	–	78	71–85	56	56–57
3 0	1	Glucose	–	–	–	–	–	–	100	–	100	–
	2	Maltose	–	–	88	–	88	–	99	–	96	–
	2	Trehalose	–	–	100	–	100	–	88	84 93	46	43–50
4 0	1	Glucose	–	–	–	–	–	–	–	–	100	–
	1	Maltose	–	–	88	–	–	–	100	–	99	–
	2	Trehalose	–	–	93	87–100	100	–	99	98–100	82	75–90
[illegible] 0	2	Trehalose	–	–	100	–	100	–	100	–	94	89–100

The localization of maltase and trehalase in the digestive tract measured in vitro

The rat intestinal tract contains much more maltase than trehalase (Table III) The enzymes are chiefly present in the small intestine The activities pres ent in the other parts of the gastrointestinal tract are low Rat pancreatic juice, obtained by a catheter had very low maltase activity (4—31 units per ml) and no detectable trehalase (less than 0 5 units per ml) The maltase is uniformly distributed along the small intestine The trehalase in contrast decreases markedly from the upper third of the small intestine to its lower third, the latter value being 10 % of the former

The intestinal disaccharidases are known to exert their physiological action inside the mucosal cells (DAHLQVIST and BORGSTROM 1961 DAHLQVIST and BRUN 1962)

Table III *Maltase and trehalase activities of the various segments of the gastrointestinal tract of the rat measured in vitro in homogenates*

Segment	No of animals	Total units of maltase activity		Total units of trehalase activity	
		Mean	Range	Mean	Range
Stomach	6	30	6–45	2	0–3
Upper third small intestine	3	700	581–845	165	116–216
Middle third small intestine	3	649	545–746	77	73–80
Lower third small intestine	3	695	593–747	15	8–22
Caecum	3	8	7–9	1	0–3
Remainder of colon	3	8	3–13	1	1–2
Caecal contents	2	1	0–2	0	–

One unit of maltase or trehalase hydrolyses 1 mg of maltose or trehalose per hour (37° C, 0 028 M substrate pH 6,5 for maltase and pH 6 0 for trehalase)

Can significant amounts of maltose or trehalose be absorbed from the gastrointestinal tract without hydrolysis?

To be able to calculate the rate of hydrolysis of the disaccharides *in vivo* we had to know whether some of the disaccharide that had been absorbed had escaped hydrolysis in the intestinal wall For this purpose we assayed the amount of sugar excreted in the urine which was collected for 2 days after the peroral administration of the disaccharide — polyethylene glycol solution For comparison we also collected the urine from rats which had been given 50 mg of each disaccharide intraperitoneally as a 10 % solution

After the intraperitoneal injection of trehalose 84—93 % of the sugar was recovered in the urine This means that trehalose like some other disaccharides such as sucrose and lactose (VERZAR and McDOUGALL 1936 DAHLQVIST and THOMSON 1963) cannot be metabolized when given parenterally and if some of this sugar is absorbed from the gastrointestinal tract without hydrolysis it should be excreted in the urine After the peroral feeding of 800 mg of trehalose however less than 3 % of the sugar was recovered in the urine This indicates that the trehalose is virtually completely hydrolyzed during the passage through the intestinal wall

Maltose in contrast was metabolized to a large extent after intraperitoneal injection Only 8—22 % of the injected amount of this sugar was recovered in the urine This is in good accord with earlier observations (VERZAR and McDOUGALL 1936) Therefore although after the peroral feeding of 800 mg of maltose less than 3 % was recovered in the urine we cannot conclude that maltose is completely hydrolyzed during absorption

LARNER and McNICKLE (1955) have recovered small amounts of maltose in the portal blood of rabbits after the peroral feeding of this sugar However

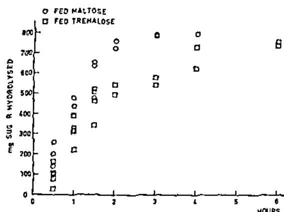


Fig 8 The course of the hydrolysis of 800 mg of maltose or trehalose after the intragastric administration of one of these disaccharides

about 95 % of the sugar recovered in the portal blood was free glucose CHAIN *et al* (1960) found only free glucose on the serosal side of isolated rat small intestine during the absorption of a starch hydrolysate The high maltase activity of the intestinal wall compared with the rate of absorption of maltose found in our experiments also makes it improbable that any significant amounts of the maltose can have passed the intestinal wall without hydrolysis

The localization of the hydrolysis of maltose and trehalose in vivo

Only small amounts of the sugars were hydrolyzed in the stomach, because the gastric contents contained only very small amounts of glucose when the disaccharides were fed (Fig 6 and 7) and the gastric absorption of sugar was unimportant (Table II) The gastric hydrochloric acid and the weak disaccharidase activities of the stomach are therefore insignificant in the hydrolysis of maltose and trehalose by the gastrointestinal tract

When maltose was fed little unhydrolyzed disaccharide reached the lower segments of the intestine (Fig 6) In the portions which reached the lower third of the small intestine after the first half hour 72 % or more of the sugar had been absorbed (Table II) and about half of the remainder had been hydrolyzed (Fig 6) The major site of the hydrolysis of maltose was the upper two-thirds of the small intestine

When trehalose was fed very little free glucose was found in the various segments As much as 20 % of the amount of trehalose fed reached the large intestine unhydrolyzed (Fig 7) This was also reflected in the low absorption indices for trehalose in the samples recovered from the lower segments of the intestine

Thus a considerable fraction of the trehalose escaped hydrolysis in the upper part of the small intestine where the trehalase activity is concentrated This fraction was slowly absorbed in the lower third of the small intestine and in the large intestine since the absorption of trehalose proceeded to completion

Table IV Comparison of the rates of hydrolysis of maltose and trehalose in vitro and in vivo

Sugar	*In vitro* (mg glucose formed/rat/hr) Mean ± S E	*In vivo* (mg glucose formed/rat/hr) Mean ± S E
Maltose	2 090 ± 147	378 ± 37
Trehalose	271 ± 26	251 ± 39

The low trehalase activity of these parts of the gastrointestinal tract may explain the slower absorption of trehalose occurring later than 1 1/2 hr after intubation

Comparison of the rates of hydrolysis of maltose and trehalose in vitro and in vivo

The course of the hydrolysis of maltose and trehalose in the intact rats calculated with the assumption that all the sugar that had disappeared from the gastrointestinal tract had also been completely hydrolyzed is seen in Fig 8 During the first 1 1/2 hr the two disaccharides were hydrolyzed at approximately the same rates but then the hydrolysis of trehalose proceeded considerably more slowly than that of maltose

The mean amounts of maltase and trehalase in the entire gastrointestinal tracts of three fasting rats determined *in vitro* with tissue homogenates are seen in Table IV These values are compared with the rates of hydrolysis of the disaccharides *in vivo* calculated from the amounts of disaccharide recovered during the first hr after intubation

It is clear that the amount of maltase in the intestinal tract greatly exceeds the activity utilized during absorption As the rates of absorption of glucose and maltose are virtually equal the availability of maltase is not rate-limiting for the absorption of this disaccharide

The rate of absorption of trehalose *in vivo* closely approaches the rate of its hydrolysis by the homogenized gastrointestinal tract under optimal conditions *in vitro* After the initial 1 1/2 hrs of absorption therefore it seems likely that it is the low trehalase activity of the lower part of the gastrointestinal tract which is the rate limiting factor in the absorption of trehalose

Discussion

One of the most striking features of the present investigation is that the rates of absorption of the three sugars studied maltose trehalose and glucose are similar during the first two hours of absorption (the "linear absorption period) The rate of sugar absorption during this period also equals the rate of absorp-

tion of sucrose and invert sugar when studied under the same conditions (DAHLQVIST and THOMSON 1963) The rates of hydrolysis of the three disaccharides by mucosal extracts *in vitro* are markedly different We have obtained evidence that these disaccharides are completely hydrolyzed during absorption Therefore the rate of hydrolysis of maltose trehalose and sucrose does not limit the rate of their absorption in the rat during the linear absorption period' The rate and site of absorption of glucose found by us agrees well with that found by REYNELL and SPRAY (1956 b)

In this study maltose was absorbed in the same location as glucose, the proximal two thirds of the rat small intestine In the human, in contrast, maltose has been found to be absorbed at a considerably lower level in the small intestine than glucose (DAHLQVIST and BORGSTROM 1961)

Regarding the rate and site of absorption after the first two hr period, there are striking differences between trehalose and the other two sugars studied Trehalose is absorbed more slowly during the later phase of the absorption process and, in contrast to glucose and maltose a considerable of the trehalose reaches the lower segments of the gastrointestinal tract This is easily explained by the relative amounts and distributions of maltase and trehalase in the various gut segments When measured *in vitro*, the maltase is present in great excess in comparison with the amount needed for the hydrolysis of maltose during its absorption *in vivo* Also, the maltase activity is high in all parts of the small intestine When the amount of trehalase is measured *in vitro* the activity found is just sufficient for the hydrolysis of the amount of trehalose that is absorbed during the linear absorption period Furthermore the maximum trehalase activity is distinctly localized to the upper third of the small intestine That fraction of the trehalose which escapes absorption and hydrolysis in the upper part of the small intestine and reaches the lower segments of the gut is then hydrolyzed and absorbed very slowly due to the low trehalase activity in these segments In spite of this no absorption of trehalose occurs without simultaneous hydrolysis as no trehalose is found in the urine

If one considers the disaccharide absorption mechanism to consist of at least three steps the entry of the disaccharide into the mucosal cells its hydrolysis and the exit of the monosaccharides into the portal blood it can be postulated that in the rat it is the third step which is rate limiting for maltose and sucrose This would also apply to trehalose absorption in the proximal third of the small intestine The similarity of the total rates of absorption in spite of the marked differences in the rates of hydrolysis of these disaccharides by mucosal extracts can be explained by this reasoning In the lower part of the intestine however the availability of trehalase seems to be a rate limiting factor in the absorption of its substrate

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Primary Afferent Depolarization Evoked from the Sensorimotor Cortex

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Abstract

CARPENTER D A LUNDBERG and U NORRSELL *Primary afferent depolarization evoked from the sensorimotor cortex* Acta physiol scand 1963 59 126—142 — Stimulation of the sensorimotor cortex evokes dorsal root potentials (DRP) in the lumbo-sacral cord Primary afferent depolarization is evoked in group Ib and group II muscle afferents and cutaneous afferents but not in Ia afferents The effects are mediated by the pyramidal tract There is spatial facilitation between the paths from primary afferents and cortex It is concluded that the pyramidal tract acts on primary afferents by giving excitatory action to interneurones of spinal reflex arcs to primary afferents

Activity in the pyramidal tract causes widespread activation of interneurones in the dorsal horn and intermediary region (LLOYD 1941 LUNDBERG NORRSELL and VOORHOEVE 1962) A recent detailed analysis has revealed facilitation from the pyramidal tract of interneurones transmitting actions to motoneurones from Ia Ib and flexor reflex afferents (FRA) (LUNDBERG and VOORHOEVE 1962) *Findings during this investigation gave rise to the question whether the* pyramidal tract also had effect on primary afferents or on the spinal reflex paths to primary afferents The reults of a systematic investigation of this problem will be presented below A basis for the present work has been the recent analysis of spinal reflex paths to primary afferents Ia afferents are depolarized by volleys in Ia and Ib afferents mainly from flexors (ECCLES MAGNI and WILLIS 1962 a) Ib afferents are depolarized by Ib volleys from flexor and extensor muscles (ECCLES SCHMIDT and WILLIS 1962 d) whereas

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cutaneous afferents receive depolarization from Ib afferents and from the FRA (ECCLES KOSTYUK and SCHMIDT 1962 c)

A preliminary report of the work presented below has been given (CARPENTER LUNDBERG and NORRSELL 1962) and similar findings have also been reported in a preliminary communication by ANDERSEN ECCLES and SEARS (1962)

Methods

The experiments were made on 17 cats under pentobarbital sodium chloralose or chloralose urethane anaesthesia The spinal cord was exposed from L4 to S1 and the L6 L7 and S1 ventral roots sectioned on the left side The following left nerves were usually dissected and mounted for stimulation or recording quadriceps (Q) posterior biceps semitendinosus (PBSt) anterior biceps-semimembranosus (ABSm) gastrocnemius-soleus (G-S) plantaris (Pl) flexor digitorum longus (FDL) deep peroneal (DP) sural (Sur) superficial peroneal (SP) posterior joint (J) In one experiment the nerves were dissected on both sides and bilateral section of the ventral roots L6 S1 was made The sensorimotor cortex was exposed on the right side and stimulation was performed as described by LUNDBERG and VOORHOEVE (1962)

For recording of dorsal root potentials the caudal filament of the L6 dorsal root was sectioned dissected free to the point of entry into the cord and mounted on recording electrodes one close to the spinal cord and the other at the cut end The interelectrode distance was about 15 mm The incoming afferent volley and slow cord dorsum potentials were recorded with an electrode placed on the dorsal root entry zone in L7

Intraspinal excitability measurements were made as described by WALL (1958) and ECCLES *et al* (1962a) Conventional glass capillary microelectrodes filled with 3 M NaCl solution and a resistance of 1—2 MΩ were used During insertion of these electrodes into the spinal cord they were used for recording of the extracellular fields whereby the appropriate location of the electrode tip for the required testing situation was assured The stimulus was either condenser discharges with a half decay of 75 μsec or square wave pulses with a duration of 0 1—0 2 msec supplied to the electrode via an isolation transformer or a radiofrequency isolation unit Intracellular recordings from primary afferents were made with 3 M KCl microelectrodes with a resistance of 5—10 MΩ After intracellular recording the extracellular field potentials were always recorded after slight withdrawal of the electrode from the axon The time constant of the amplifiers used for recording was 0 8 sec

Results

1) *Dorsal root potentials*

On stimulation of the sensorimotor cortex a dorsal root potential (DRP) is evoked (Fig 1 record G—H) which resembles the DRPs evoked from the peripheral nerves For comparison the DRPs evoked from different peripheral nerves are shown in A—D The DRP in A was evoked by a single group I volley from the Q nerve The additional effect in B was evoked from high threshold muscle afferents A group I train from Q caused the DRP shown in C and a single volley in the SP nerve the effect in D The DRP evoked from these different afferent systems resemble closely those found in the spinal preparation (BROOKS and FOURTES 1952 ECCLES *et al* 1962 a ECCLES KOSTYUK

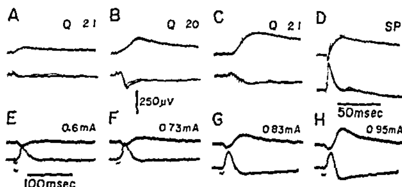


Fig 1 The upper traces are dorsal root potentials (DRP) recorded from the most caudal dorsal root filament in L6 The lower traces were recorded from the dorsal root entry zone in L7 The quadriceps nerve (Q) was stimulated in A—C and the stimulus strengths are given in multiples of threshold strength for the nerve A shows the effect of a single maximal group I volley and the additional DRP in B is due to activation of high threshold muscle afferents The DRP in C was caused by a group I train from Q and D shows the effect of a single volley in the cutaneous superficial peroneal nerve (SP) In E—H the sensorimotor cortex was stimulated at the indicated strength In the DRP recording an upwards deflexion signals negativity of the electrode close to the spinal cord In the lower traces an upwards deflexion singals negativity of the electrode in contact with the cord dorsum Records A—D consist of superimposed traces and E—H of single traces Records C and D were taken at the faster speed shown in D and all the other records at the slow speed Calibration refers to the dorsal root potential

and SCHMIDT 1962 b) This is of importance for the present analysis because some actions to primary afferents are suppressed in the decerebrate state (CARPENTER ENGBERG FUNKENSTEIN and LUNDBERG 1963) Repetitive stimulation is usually required to evoke a DRP from the sensorimotor cortex but on a few occasions a small effect was evoked by a single strong stimulus The latency from the onset of cortical stimulation was usually about 25 msec Cortical stimulation evokes a negative cord dorsum potential after 8—10 msec and associated with this potential there is a positive DRP (E) At stronger stimulation a negative DRP and a positive cord dorsum potential appears in parallel (F—H) The initial small positive DRP resembles the DR IV of LLOYD and McINTYRE (1949) and it has been shown that the DR IV is not a trans membrane potential (ECCLES and KRNJVIC 1959) In some of the preparations anaesthetized with chloralose and chloralose urethane the cord dorsum potential has reversed to an initial positivity at stronger cortical stimulation This initial positive cord dorsum potential had the same latency as the negative cord dorsum potential evoked at weaker strength of cortical stimulation and resembles the potential resulting on stimulation of the brain stem (CARPENTER ENGBERG and LUNDBERG 1962)

The cortical area from which DRPs can be evoked in the lumbar region is shown in the drawing in Fig 2 Records B and D in Fig 2 illustrate that no DRPs can be evoked after section of the pyramid hence the effects in A re corded from the left dorsal root filament and in C recorded from the right

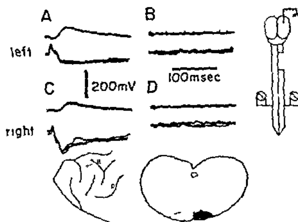


Fig 2 The effect of stimulation of the right sensorimotor cortex before (A and C) and after (B and D) section of the pyramid (lower right diagram). Recording as in Fig 1 but on the right and left side as indicated in the diagram. Each record consists of 3—4 superimposed traces. Stimulation within the shaded area in the lower left hand diagram yielded the largest effects. The symbols in this diagram denote A s cruciatus B s ansatus C s lateralis D s suprasylvius E s coronalis F s præsylvius

dorsal filament are mediated exclusively via the corticospinal tract. The records in Fig 2 also illustrate that bilateral effects are evoked and since in this experiment the cord was hemisectioned it can be concluded that the pyramidal tract has bilateral actions in the spinal cord. This may be related to the fact that volleys in dorsal roots or peripheral nerves have bilateral actions (BARRON and MATTHEWS 1938, LLOYD and MCINTYRE 1949). If the stimulus strength after pyramidal section was increased above 1.5 mA a DRP and an initial positive cord dorsum potential did appear. At this high strength a stimulus escape to subcortical structures cannot be entirely excluded. However in preparations with intact pyramids, an initial positive cord dorsum potential was sometimes found at lower strength of cortical stimulation (cf. above). Since strong stimulation of the dissected pyramid gave the same effect as that shown in record H Fig 1, it is likely that of the effects evoked from cortex those associated with an initial positive cord dorsum potential may be mediated via other paths than the corticospinal tract. When not otherwise mentioned the actions referred to below are assumed to be mediated by the corticospinal tract.

2) *Types of afferent fibres depolarized from cortex*

In one series of experiments a stimulating electrode was inserted into the gray matter in either the dorsal or the ventral horn and the test volley was recorded in the peripheral nerve (WALL 1958). The effect of conditioning cortical and peripheral stimulation were compared. In another series of 7 experiments intracellular recordings were made from single axons permitting a direct measurement of the depolarization evoked from different sources.

Ia afferents. In the experiment of Fig 3 the stimulating electrode was inserted in the G—S motor nucleus. The test volley recorded in the G—S nerve could be facilitated by group I volleys from the PBSt and DP nerves as is shown in

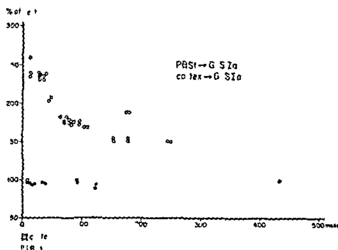


Fig 3 The effect of cortical stimulation on the excitability of presynaptic terminals of Ia fibres The testing stimulus was deliver d through a microelectrode inserted into the motor nucleus of gastrocnemius soleus (G—S) at the site where the maximal Ia focal potential could be re corded The test discharge was recorded in the G—S nerve. 100 % on the ordinate represents the height of the unconditioned test discharge

Cortical stimulation did not increase the height of the test discharge (●) but there was a marked effect by a train of 4 group I volleys from PBSt (○) The conditioning stimuli are indicated below the abscissa

the Fig for a train of 4 group I volleys from PBSt However in no case was there any trace of facilitation when cortical stimulation was used for conditioning In other experiments intracellular recording was made from 16 identified Ia fibres In none of them did cortical stimulation cause any trace of depolarization whereas large actions were usually obtained from the PBSt and DP nerves (cf ECCLES *et al* 1962 a)

Ib afferents The effects on Ib fibres were investigated with the testing stimulating electrodes inserted into the intermediate nucleus of CAJAL. With this location of the stimulating electrode stimulation of the sensorimotor cortex did give increased excitability in group I fibres It seemed likely that this effect was exerted in Ib fibres and this was proven by the use of the collision technique in preparations in which group I volleys displayed separation in Ia and Ib components (ECCLES ECCLES and Lundberg 1957 LAPORTE and BESSOU 1957) Stimulating electrodes were placed on the nerve from which the test volley was recorded and a maximal Ia volley from the periphery was timed to collide with the outgoing group I volley evoked by the test stimulus applied in the intermediary region Hence a pure Ib volley could be recorded in the periphery Record A in Fig 4 shows the maximal group I volley from G—S display separation in Ia and Ib components and B shows the double volley test where the first volley is maximal for Ia Record C shows the size of the test volley recorded in the G—S nerve when not decreased by a colliding volley D shows

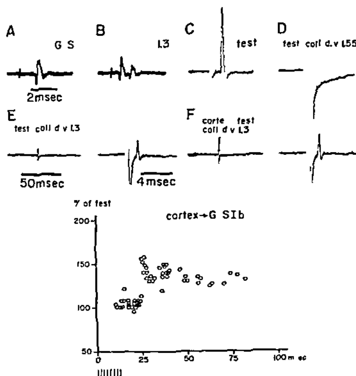


Fig 4 Effect of stimulation of the sensorimotor cortex on the excitab lity of presynaptic terminals of Ib fibres The testing stimulus was deli ered through a microelectrode inserted into the inter mediary nucleus at the site where the maximal Ib focal potential could be recorded The discharge was recorded in the G—S nerve Through another (cent al) pair of electrodes on the G—S nerve a centripetal group I volley could be evoked and this volley vas timed to collide with the centrifugal group I volley Reco d C shows the discharge evoked from the intraspinal microelectrode in the absence of any centripetal colliding volley In D a maximal group I colliding volley was given In this preparation the G—S group I volley dispalyed the separation in Ia and Ib components (reco d A) In B the double volley test was employed to show that the Ia volley was maximal at a st ength of 1 3 times threshold In record E (shown at two sweep speeds) a maximal Ia coll d ng volley was used hence the outcoming impulses are in Ib fibres and the height of this volley could be used as a measure of excitabil ty in the p esynaptic terminals of Ib fibres The effect of co t al stimulation is shown in F and in the graph below

the disappearance of the test volley with a maximal group I colliding volley In E a maximal Ia colliding volley was used and hence the records (at two speeds) show a test Ib volley The facilitatory effect of conditioning cortical stimulation is shown in F and the time course of the effect is shown in the graph in Fig 4 Similar findings were made with intracellular recording Records A—C Fig 5 serve to identify a Ib fibre from ABSm D shows the depolarization evoked on repetitive group I stimulation of the Q nerve and E the depolarization evoked from cortex The depolarization in E is unusually large but was associated with an initial positive cord dorsum potential Hence other pathways than the corticospinal tract may have mediated the action Generally the effect

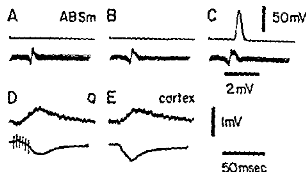


Fig 5 Intracellular recording from a group Ib fibre of the ABSm nerve (upper traces) Records A—C were obtained at increasing strength of stimulation of the ABSm the strength in C being almost maximal for Ib The lower traces in A—C and the middle traces D and E were recorded from the dorsal root entry zone in L7 The lower traces D and E were recorded when the microelectrode had been withdrawn to a just extracellular position In the microelectrode recording positivity is upwards and in the surface recording positivity is downwards Record D shows the depolarizing effect of a group I train in the Q nerve and the effect of cortical stimulation is is shown in E

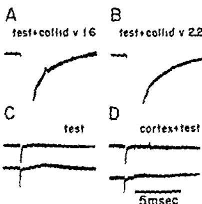


Fig 6 Effect of cortical stimulation on the excitability of the presynaptic terminals of a group II fibre A stimulating electrode was inserted into the dorsal horn of the gray matter and recording was made from the nerve to G—S As in Fig 4 a colliding volley could be evoked from another pair of electrodes on the G—S nerve In A a spike persists when a maximal group I colliding volley was evoked at a strength of 1 6 times threshold but disappeared in D when the colliding volley was evoked at 2 2 times threshold so that the larger group II fibres were also activated In C the intraspinal stimulus strength was decreased to a value just subthreshold for the fibr Following cortical stimulation (6 stimuli 55 msec earlier) the same stimulus regularly did excite the fibre (D) The lower traces in C and D were recorded from the S1 dorsal root entry zone

as much smaller than that evoked from the PBSt or Q nerve and in many cons there was only a trace of depolarization on strong cortical stimulation record H Fig 10)

Group II muscle afferents A stimulus applied through an electrode inserted into the dorsal horn will also give rise to spikes recorded after a longer latency than the one found for group I impulses These spikes can still be recorded in the periphery when a maximal group I volley from the periphery was evoked to collide with the test volley (record A Fig 6) but disappeared when the peripheral stimulus strength was raised to activate also the slower fibres in record B at 2 2 times threshold (1 6 being maximal for group I) The conduction velocity of the fibre in Fig 6 was 52 m/sec hence it can be classified as a group II fibre The relation between conduction velocity and stimulus strength corresponds to that found by ECCLES and LUNDBERG (1959) In C the strength of the test stimulus was lowered so that it was submaximal for activation of the fibre Following cortical stimulation the same test stimulus regularly evoked a spike (D) 12 group II fibres were tested in this manner and 6 of them displayed in

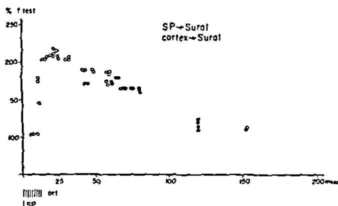


Fig 7 Effect from the sensorimotor cortex on the excitability of large cutaneous afferents The testing stimulus was delivered through a microelectrode inserted into the dorsal horn and the discharge was recorded in the sural nerve The graph shows the facilitatory effect of a single volley in the cutaneous superficial peroneal (SP) nerve (○) and of cortical stimulation (●) The conditioning stimuli are indicated below the abscissa

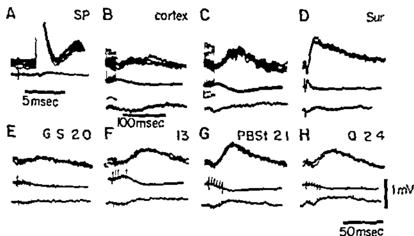


Fig 8 Intracellular recording as in Fig 5 but from a large cutaneous afferent of the superficial peroneal nerve (SP) The spike evoked from the SP nerve is shown at high amplification in A. B and C show the effect of weak and strong stimulation of the sensorimotor cortex The effects in D—H were evoked on stimulation of peripheral nerves The sural nerve (Su) was stimulated in D E shows the effect of a group I train from G—S and in F the G—S nerve was stimulated at a strength of 13 times threshold for the nerve The primary afferent depolarizations in G and H were evoked by maximal group I trains in the PBSt and Q nerves respectively

The intracellular records (upper traces) and the surface records (middle traces) consist of many superimposed traces but the field potentials recorded after withdrawal of the microelectrodes to a just extracellular position (lower traces) are single traces

creased excitability after stimulation of the sensorimotor cortex. Hence there is evidence that these afferents also are depolarized from cortex

Cutaneous afferents In order to investigate excitability changes in cutaneous afferents the stimulating electrode was inserted into the dorsal horn to a

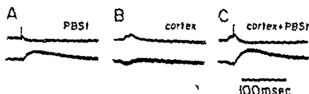


Fig 9 The lower traces were recorded from the most caudal dorsal root filament in L6 and the upper traces from the dorsal root entry zone in L7 Record A shows the effect of a single group I volley from PBSt In B the contralateral sensorimotor cortex was stimulated at a strength liminal for evoking a DRP C shows the effect of combined stimulation of PBSt and cortex The records consist of 3—4 superimposed traces

Fig 10 Intracellular recording (upper traces) from a group I afferent of the plantaris (Pl) nerve (record K) The middle traces in A—F and H—J are triphasic recordings from the dorsal root entry zone in L7 The lower traces in these records were recorded when the microelectrode had been withdrawn to a just extracellular position In G the additional lowest record was obtained from the dorsal root entry zone simultaneously with the extracellular microelectrode trace shown just above in the same record Records A—F show the effect of maximal group I trains from the muscle nerves indicated in each record G shows the effect of repetitive stimulation of the cutaenous sural nerve and H the effect of stimulation of the contralateral sensorimotor cortex The effect of a single maximal group I volley in the G—S nerve is shown in I and in J in combination with cortical stimulation at the strength used in H Time calibration 50 msec refers to records A—F and the lower two traces in G Records H—J and the upper two traces in G were taken at the slowest sweep speed All records consist of 3—4 superimposed traces

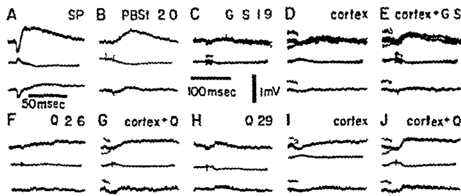


Fig 11 Intracellular recording (upper traces) as in Fig 5 and 8 but recording from a large cutaneous axon of the sural nerve The effect of repetitive stimulation of the cutaneous SP nerve is shown in A and B shows the effect of a group I train in the PBSt nerve A maximal group I G—S train has no effect in C but gives a primary afferent depolarization in E when combined with stimulation of the contralateral sensorimotor cortex The effect of cortical stimulation alone is shown in D The corresponding records in H—J show spatial facilitation between single volleys in high threshold muscle and cortical stimulation. The Q nerve was stimulated in H and J at a strength of 79 times threshold for the nerve In F and G the Q nerve was stimulated at ? 6 times threshold for the nerve (maximal for group I) and there is no facilitation from cortex of the effect from a single group I volley in the Q nerve (record G) Record A and B were taken at the fast and the other records at the slow speed The intracellular records in C—E and the corresponding records from the dorsal root entry zone consist of superimposed traces, but all other records are single traces

position where the maximal N_1 field potential could be recorded (COOMBS CURTIS and LANDGREN 1955) The curves in Fig 7 show the effects of conditioning stimulation of the SP nerve (○) and of cortex (●) on the height of the test spike recorded in the sural nerve Cortical stimulation gives an increase of the test discharge that is almost as large as that obtained from the cutaneous nerve Similar findings were made with intracellular recording from cutaneous afferents and are illustrated in Fig 8 B and C show the effects of weak and strong cortical stimulation D—H show the effects evoked from the periphery D shows the large effect evoked by a single volley in the sural nerve and G and H the depolarization given by a group I train from the PBSt and Q nerves respectively The depolarization in F was caused by impulses in high threshold muscle afferents from G—S for comparison the effect of a group I train is shown in E These effects from the periphery confirm the pattern described by ECCLES *et al* (1962 c)

3) *Interaction between effects evoked from cortex and the periphery*

Since it has been postulated that volleys in the pyramidal tract exert actions on motoneurones through excitation of interneurones of the spinal reflex paths it was of interest to investigate whether there is a similar organization for the actions on primary afferents For this purpose spatial facilitation and depression between effects from the two sources have been investigated

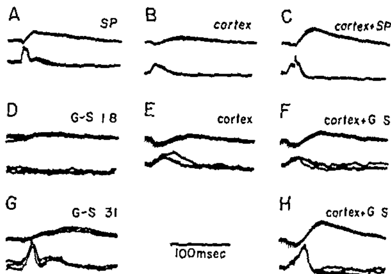


Fig 12 *The upper traces were recorded from the most caudal dorsal root filament in L6 and the lower traces from the dorsal root entry zone in L7* The superficial peroneal (SP) nerve was stimulated in A and C at a strength of 1 1 times threshold B shows the effect of cortical stimulation alone and C of combined stimulation of cortex and the SP nerve Records D—H were obtained in another experiment The G—S nerve was stimulated at a strength of 1 8 times *threshold (just maximal for group I) in D and F and at 31 times threshold in G and H* E shows the effect of cortical stimlation alone and F and H of combined stimulation of cortex and the G—S nerve nerve at the strengths that were used to obtain the corresponding records in D and G All records consist of superimposed traces

Spatial facilitation It was a regular finding that stimulation of the sensorimotor cortex facilitates the DRP evoked from the periphery Facilitation of group I actions are shown in Fig 9 A shows the DRP evoked by a maximal group I volley from PBSt and in B cortex was stimulated at a strength barely supraliminal for the appearance of a DRP With combined stimulation in C there is a marked spatial facilitation Eccles *et al* (1962 a) have shown that Ia and Ib volleys contribute to the DRP evoked from flexor nerves We have confirmed this finding and investigated effects from cortex on the DRPs from these afferent systems *In no case did we observe any facilitation of the DRP evoked by a* group Ia train but when the stimulus strength was raised to activate Ib fibres the DRP could regularly be facilitated from cortex There was also facilitations of the DRP evoked by group I volleys from extensors (Fig 12 D—F) Volleys in group I muscle afferents can evoke depolarization in Ia and Ib muscle afferents and in cutaneous afferents *The further analysis was therefore made* with intra axonal recording from these different types of afferents The records in Fig *10* are from a plantaris *Ib* axon (K) group *I actions are drawn from* extensors and flexors (Eccles *et al* 1962 d) and there is only a small DRP evoked by a volley in a cuntaneous nerve (G) Cortical stimulation gave only a very small depolarization in this axon (H) but there was a very considerable facilitation of group I effects from cortex as is shown for the effect from G—S

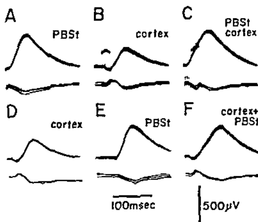


Fig 13 Depressive interaction of the DRPs evoked from cortex and by group I volleys from PBSt Records A and E show the effect of a group I train in the nerve to PBSt Records B and D show the effect of stimulation of the contralateral sensorimotor cortex Combined stimulation was used in C and F as indicated in the records All records consist of 3—4 superimposed traces

in I and J Intra axonal recording from cutaneous afferents likewise revealed cortical facilitation of group I actions This is shown for an axon of the sural nerve in Fig 11, A and B show the effect of repetitive stimulation of the SP and PBSt nerves respectively A group I train from G—S did not evoke any depolarization in C but following cortical stimulation in E the same G—S train was effective Intracellular recording was also made from 12 Ia fibres but in none of them could the depolarization evoked from PBSt or DP be facilitated from cortex

It was regularly found also that the DRPs evoked from the FRA could be facilitated from cortex This is illustrated for the DRP evoked from cutaneous afferents in A—C Fig 12 and for the DRP from high threshold muscle afferents in G and H ECCLES *et al* (1962 c) have shown that cutaneous afferents are depolarized not only from group I muscle afferents but also from the FRA (cf also Fig 7) With intracellular recording from cutaneous afferents it was found that cortical stimulation facilitated the effect evoked from cutaneous afferents high threshold muscle and joint afferents (Fig 11 D—F)

Depression The DRP evoked from cortex occludes with the DRP evoked either from group I afferents or from cutaneous afferents In Fig 13 A—C PBSt stimulation precedes the cortical stimulation and D—F illustrates the reverse situation with conditioning of cortex and testing PBSt It is of interest that in each case the time course of the depression is of the same order ECCLES *et al* (1962 c) discussed the interaction of the DRPs from two peripheral sources in terms of 1) The depolarizing action on primary afferents which will depress transmission from these afferents 2) Depression due to the usage of a common interneuronal line If in the present case 1) was a dominating factor the reciprocity of the effect raises the question of whether the terminals of the pyramidal tract are depolarized from the peripheral nerve These findings will be subject to a special analysis, meanwhile other possibilities to account for this depression must be considered We do not know to what extent the receptive subsynaptic membrane of the primary afferents can be depolarized If the fibres can only be depolarized to a level slightly below the resting potentials then a depression after a conditioning volley could be due

to the depolarization in the receiving fibres Another explanation for the depression in Fig 13 could be inhibitory interaction at an interneuronal level between the reflex paths to primary afferents Further experiments have revealed that cortical stimulation has an inhibitory action on the path from Ia to Ia (LUNDBERG and VYKLICKY 1963) This action is probably exerted through activation of FRA interneurones and may be due to depolarization of terminals of interneurones

Discussion

In a previous investigation of the mechanism by which the pyramidal tract exerts spinal actions the question was raised whether effects also are exerted on primary afferents The finding that stimulation of the sensorimotor cortex gives a large DRP shows that a depolarization of primary afferents does occur The cortical area from which effects in the lumbar region are obtained is the one from which hind limb movements can be evoked (cf LIVINGSTON and PHILLIPS 1957) and from which excitatory actions are exerted on interneurones supplying synaptic actions to hind limb motoneurones (LUNDBERG and VOORHOEVE 1962) The disappearance of the DRPs after section of the pyramid shows that the effect is mediated by the corticospinal tract, but evidence has been presented that in cats anæsthetized with chloralose effects from cortex can be evoked via other paths Further analysis with intra axonal recording and with intraspinal excitability measurement (cf WALL 1958) revealed depolarization in Ib afferents and also in cutaneous afferents and group II muscle afferents The two latter systems of afferents are part of the FRA and presumably all afferents belonging to the FRA are depolarized from cortex There was on the other hand never any indication of depolarization in Ia afferents

ECCLES et al (1962 a) have shown that there is a close parallelism between primary afferent depolarization and presynaptic inhibition i e the depression of the synaptic actions exerted by these afferents For this reason it is postulated that cortical stimulation gives presynaptic inhibition of transmission to nerve cells excited from Ib fibres and from the FRA LUNDBERG and VOORHOEVE (1962) searched for presynaptic inhibition from cortex to the Ia pathway to motoneurones It is in agreement with the present results that no effect was observed in 21 out of 22 motoneurones tested However in one motoneurone a depression of the Ia EPSP was found This may have been a stray action from the pyramidal tract or the effect may be secondary to activation of brain stem centres from which depolarization in Ia fibres can be evoked (CARPENTER et al 1962) and the possibility of an effect exerted via this system cannot be excluded

The experiments revealing marked spatial facilitation between cortical and peripheral stimuli strongly suggest that the effects from cortex are exerted via interneurones of the spinal reflex paths to primary afferents The DRP evoked by stimulation of group I muscle afferents from flexors and extensors can be

facilitated from cortex and the same holds true for the effects from the FRA. As would be expected (cf ECCLES *et al* 1962 c) further analysis revealed that the action from the FRA on to cutaneous afferents can be facilitated from cortex However, the facilitation of effects evoked from group I afferents required a more detailed investigation because group I volleys can depolarize Ia and Ib afferents as well as the FRA With intra axonal recording it was found that cortical stimulation facilitated the path from group I to Ib afferents and to cutaneous afferents but we were not able to find facilitation of group I actions to Ia afferents Likewise there was no facilitation from cortex of the DRP evoked by Ia volleys from flexors which presumably depolarize only Ia afferents (ECCLES *et al* 1962 a) In this connection it is of interest that LUNDBERG and VOORHOEVE (1962) failed to find any facilitation from cortex of the depres sion of motoneuronal EPSPs evoked by group I volleys from flexors (cf FRANK and FUORTES 1957, ECCLES ECCLES and MAGNI 1961)

Support for the postulate that the pyramidal tract gives excitatory action to the interneurones of the spinal reflex paths to primary afferents is given by the findings that convergence of excitation from the periphery and from cortex is very common on to interneurones of the dorsal horn and intermediary region This is found for interneurones activated from group I afferents from cutaneous afferents from the FRA and from group I and the FRA (LUNDBERG NORRSELL and VOORHOEVE 1962) Of all the reflex paths (with at least one interneurone) to motoneurones and to primary afferents so far tested the one to Ia fibres is the only one for which we have failed to obtain evidence for facilitatory action from the sensorimotor cortex. However the reflex path to Ia afferents is not independent of cortex Further experiments have shown that the depolarization evoked in Ia fibres by group Ia volleys from flexor nerves can be inhibited from cortex or from the FRA (LUNDBERG and VYKLICKY 1963) The possibility that this inhibitory action from cortex conceals a facilitation of the path to Ia fibres cannot be entirely exluded but does not seem likely since there was no facilita tion at weak cortical stimulation of actions in Ia fibres

What is now the physiological significance of the presynaptic action from the sensorimotor cortex? It is well known that stimulation of the motor cortex can cause inhibitory effects which in the early experiments were manifested by muscle relaxation (cf TERZUOLO and ADEY 1960) It does however not seem likely that these actions are due to depolarization of primary afferents Inhibition of motoneurones can be revealed with monosynaptic test methods and it has also been found that IPSPs can be evoked in motoneurones from cortex (PRESTON and WHITLOCK 1960 LANDGREN, PHILLIPS and PORTER 1962 LUNDBERG and VOORHOEVE 1962) Presumably these inhibitory actions are secondary to excitation of inhibitory interneurones of spinal reflex paths to motoneurones as is indicated by the finding that inhibition dominates in extensor and excita tion in flexor motor nuclei (LUNDBERG and VOORHOEVE 1962) The significance of the effect on primary afferents should be more relevant when we are con

cerned with spinal reflexes from Ib afferents and from the FRA The depolarization of primary afferents would oppose the facilitatory action on the path to motoneurones

It is of interest to consider the results of experiments on the effect on spinal reflexes of ablation of the sensorimotor cortex or of section of the pyramid There is evidence of an increased threshold for the scratch reflex and the flexion reflex after section of the pyramid (TOWER 1935) and this finding could indicate that the loss of facilitatory effect to the interneurones supplying actions to motoneurones is the more decisive factor

At first sight it may seem surprising that cortical stimulation mobilizes both the spinal reflex actions to motoneurones and primary afferents because these effects will counteract each other However, the same holds true for the spinal reflexes and this apparent contradiction should be discussed in view of the postulates made with regard to the functional significance of the spinal reflex depolarization of Ib afferents and the FRA ECCLES *et al* (1962 a, c d) have pointed out that in both cases presynaptic inhibition constitutes a negative feedback and postulated that it serves to reject stray excitation and therefore contributes to the local sign of a reflex. In view of this hypothesis it is more easy to understand the function of the cortical effect on reflex paths to primary afferents If a certain reflex path to motoneurones is mobilized from cortex it is certainly beneficial if at the same time the negative feedback serving this reflex action is also mobilized so that the local sign of the reflex is maintained

Summary

The effect of stimulation of the sensorimotor cortex on primary afferents in the lumbo-sacral cord has been investigated by recording of dorsal root potentials (DRPs) by intracellular recording from fibres and by intra-spinal excitability measurments

Stimulation of the sensorimotor cortex evokes large DRPs and associated positive cord dorsum potentials The effects are obtained from the hindlimb area and are mediated by the pyramidal tract Bilateral effects are evoked from each side

Cortical stimulation depolarizes Ib fibres group II muscle afferents and cutaneous afferents It is postulated that also other afferents belonging to the flexor reflex afferents (FRA) are receptive Cortical stimulation does not depolarize Ia fibres

Cortical stimulation facilitates the DRPs evoked by group Ib volleys from flexor or extensor nerves and from volleys in the FRA There is facilitation from cortex of the path from group I afferents to Ib and to cutaneous afferents and of the path from the FRA to cutaneous afferents but not of the path from group I to Ia afferents

It is postulated that impulses in the pyramidal tract depolarizes Ib afferents and the FRA by evoking excitatory action in the interneurones transmitting effects to them from primary afferents

The results are discussed in relation to findings concerning effects from the pyramidal tract on reflex paths to motoneurones and in relation to ideas concerning the functional significance of reflex actions to primary afferents

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Organization of Ascending Tracts in the Spinal Cord of the Duck

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Abstract

Oscarsson O I Rosen and N Uddenberg *Organization of ascending tracts in the spinal cord of the duck* Acta physiol scand 1963 59 143—153 — Discharges evoked in ascending spinal tracts on stimulation of muscle skin and mixed leg and wing nerves were recorded from dissected fascicles of the cord The results show that ascending tracts in the bird are organized as in the mammal (i) dorsal tracts are mono- and polysynaptically activated only from ipsilateral nerves (ii) ventral tracts are monosynaptically activated only from contralateral nerves and polysynaptically activated from both ipsilateral and contralateral nerves It is concluded that the former tracts are uncrossed and the latter tracts crossed at the spinal level — Low threshold muscle afferents activate two ascending spinal tracts one crossed tract related to leg nerves and one uncrossed tract related to wing nerves There is evidence that the former but not the latter tract is strongly inhibited from skin and high threshold muscle afferents It is suggested that the crossed tract is homologous to the ventral spinocerebellar tract in mammals The dorsal spinocerebellar tract either does not exist in the duck or has an organization different from that in the mammal

Some general principles in the organization of ascending spinal tracts were recently described for the mammalian spinal cord (Magni and Oscarsson 1962 b Holmqvist and Oscarsson 1963) It was found that tracts ascending in the dorsal part of the lateral funiculus are monosynaptically activated only from ipsilateral nerves whereas tracts ascending in the ventral part of the lateral funiculus and in the ventral funiculus are monosynaptically activated only from contralateral nerves These facts and histological evidence that

primary afferents almost exclusively terminate on the ipsilateral side indicate that the dorsal tracts are uncrossed and the ventral tracts crossed at the spinal level

This organization has been found in one marsupial and two placental species and may be regarded as fundamental to the mammalian spinal cord The possibility that this organization exists in other vertebrate classes has now been tested in experiments on the duck In these experiments it was also of interest to see whether any of the ascending tracts found in the mammalian cord could be recognized It has been shown that the functional organization of the spinocerebellar tracts follows constant and readily recognizable patterns in widely different mammalian species (MAGNI and OSCARSSON 1962 a)

Methods

The experiments were performed on domestic ducks of 2 3—3 2 kg which were anesthetized with urethane (1 g per kg body weight) given intraperitoneally After one half to one hour a supplementary anesthetic (thiopentone) was injected into the medial tarsal vein until a reflexless anesthesia was obtained The injections were repeated when necessary during the operation After spinalization the animals were artificially ventilated and flaxedil was given to prevent reflexes The body temperature was 37 5 to 39 C which is a few degrees below the normal body temperature

Leg and wing nerves were dissected bilaterally and mounted for stimulation in pools of warm mineral oil In the leg there were prepared the sciatic nerve and the skin nerve branching off the sciatic close after its emergence from the *foramen ischiadicum* and distributed to the dorsal aspect of the lower leg The muscle nerve innervating the medial and deep heads of the gastrocnemius muscle and the plantaris muscle (cf BRONN 1891) was sometimes dissected instead of the sciatic nerve In the wing the mixed radial nerve and a skin nerve (nervous cutaneus brachii superior BRONN 1891) were prepared

A large cervical vertebra (C5 C6 or C7) was delaminated Bleeding from extradural veins was avoided by limiting the laminectomy to one vertebra and by ligating the dorsal longitudinal vein at the rostral and caudal ends of the exposure The dura was opened and the cord transected at the rostral end of the laminectomy The dorsal funiculi were dissected free and sometimes left in continuity with the cord at the caudal end of the exposure for monitoring of the ingoing volley The remaining portion of the cord was divided in the midline and the cord halves split into fascicles of various sizes (cf Fig 1—3) and mounted on recording electrodes as described previously (LAPORTE LUNDBERG and OSCARSSON 1956) In all 36 dissected fascicles (not including the dorsal funiculi) were recorded from The cross sectional areas of the fascicles were assessed at the end of the experiments as described by HOLMQVIST and OSCARSSON (1963)

In some experiments the ingoing volley from the muscle nerve was recorded triphasically some cm proximally of the stimulating electrode and at the end of the experiment also monophasically from the cut end of the sciatic nerve Attempts to expose the spinal cord in the thoraco-lumbar region were unsuccessful because of profuse bleeding and did not permit either monitoring of ingoing volleys at the dorsal root entrance or recording from dissected fascicles of the spinal cord in this region However at the end of some experiments a small opening was made in one of the lumbar vertebrae to permit stimulation of the spinal cord and the ascending volley was recorded at the cervical level for calculation of the conduction velocity of the fastest cord fibres

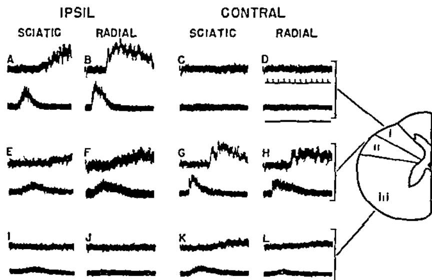


Fig 1 Discharges evoked in tracts ascending in the fascicles (i—iii) indicated in the diagram on stimulation of ipsilateral and contralateral sciatic and radial nerves Upper and lower traces were taken simultaneously at different speeds Time scales in msec Distances Stimulating electrode on sciatic nerve — spinal cord 7 cm spinal cord — recording site 29 cm Stimulating electrode on radial nerve — spinal cord 6 5 cm spinal cord — recording site 16 cm.

The stimulating and recording technique has been described before (LAPORTE *et al* 1956 HOLMQVIST and OSCARSSON 1963) Stimulus strength is usually given as multiples of the strength needed for evoking a barely visible ingoing volley (threshold strength) The strength was 15—25 times threshold when not otherwise stated The electrical events were displayed on two double beam oscilloscopes

Results

1 General pattern of ascending discharges

Stimulation of mixed leg and wing nerves produced characteristic discharges in the dissected fascicles of the spinal cord as shown in Fig 1 The general pattern of these discharges will be described in this section the discussion of synaptic linkage and relation to muscle and cutaneous afferents will be postponed to section 2

In the dorsomedial fascicle (i) discharges were evoked only by stimulation of ipsilateral nerves (Fig 1, A—D) The sciatic nerve volley produced a slowly increasing discharge without distinct front A volley in the radial nerve elicited a large monosynaptic response followed by later activity

In the dorsolateral fascicle (ii) discharges appeared on stimulation of both ipsilateral and contralateral nerves (E—H) However distinct monosynaptic discharges were only evoked from the contralateral nerves and the late activity

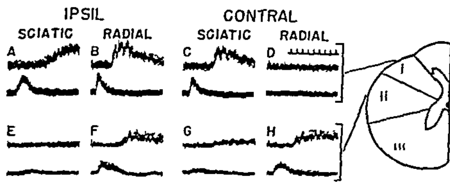


Fig 2 Discharges evoked in tracts ascending in the fascicles (i and ii) indicated in the diagram on stimulation of ipsilateral and contralateral sciatic and radial nerves Upper and lower traces were taken simultaneously at different speeds Time scales in msec. Distances Stimulating electrode on sciatic nerve — spinal cord 6 5 msec spinal cord — recording site 25 cm Stimulating electrode on radial nerve — spinal cord 9 cm spinal cord — recording site 10 5 cm

was larger when produced by volleys in these nerves The trace of early monosynaptic activity evoked from the ipsilateral radial nerve in the dorsolateral fascicle (ii) of Fig 1 was never observed when this fascicle was dissected more ventrally (Fig 2, F)

In the ventral fascicle (iii) only small discharges were observed These discharges were larger on stimulation of contralateral nerves than on stimulation of ipsilateral nerves (Fig 1, I—L) Early monosynaptic responses were only observed in some experiments as for example in Fig 3 K and L from the ..itaneous leg nerve and less distinctly from the sciatic nerve in Fig 1 K These ..onosynaptic discharges never occurred on stimulation of ipsilateral nerves

The lack of contralateral discharges in the dorsal part of the lateral fascicle was observed when the dissection was made as in Fig 1 When the dorsomedial fascicle was dissected more ventrally (Fig 2) a contralateral discharge appeared on stimulation of the leg nerve (C) but not on stimulation of the wing nerve (D) This shows that the area occupied by tracts activated exclusively from ipsilateral nerves extends further ventrally for "wing tracts than for "leg tracts

Correlation The general pattern of organization of ascending tracts in the bird spinal cord is similar to that in the mammalian cord (cf Holmqvist and Oscarsson 1963) In both classes of animals tracts in the dorsal part of the lateral funiculus are activated only from ipsilateral nerves whereas tracts ascending more ventrally in the cord are monosynaptically activated only from contralateral nerves and polysynaptically activated both from contralateral and ipsilateral nerves In both classes of animals the tracts activated exclusively from ipsilateral nerves occupy an area which extends further ventrally for "forelimb tracts than for "hindlimb tracts

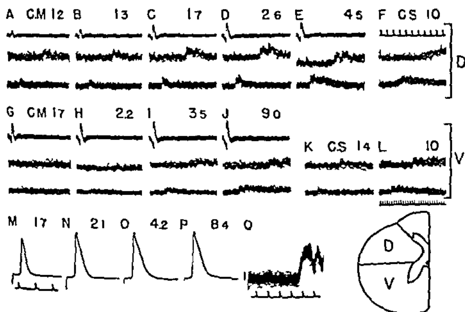


Fig 3 Records A—L show discharges evoked in tracts ascending in the dorsal (D) and ventral (V) fascicles nd cated in the diagram on st mulation of contralateral muscle (C. M) and skin (C S) nerve in the leg Stumulus st engths relative to thresl old of ingoing volley are indicated Upper traces (A—E and G—J) show the ingoing volley recorded triphasically from the sc at c nerve 4 0 cm prox mally of st n ulating electrode on muscle nerve middle and lower traces (A—L) show th ascending discharges at different speeds Time scales in msec D stances Stimulating el ctrod on muscle nerve — sp nal cord 7 0 cm stimulating electrode on skin nerve — spinal cord 3 5 cm spinal cord — recording site 27 cm.

Records M—P show monophasic ecording from cut end of sciatic nerve of volley evoked by stimulation of the muscle nerve at ind cated strengths Distance st mulating electrode — re ording electrode 3 8 cm Time scale in msec

Record Q shows olley in spinal fibres evoked by stimulation of the cord in the lumba region and recorded at th fifth cervical segment from the dissected lateral f in culus Distance between stimulating site and recording s te 3? cm T me scale n msec

2 *The organization of certain tracts*

a Leg tracts A detailed analysis of the various components of the dis charges was hampered by the small size of the responses that were recorded on stimulation of individual nerves It was however possible to study some of these components more closely

Fig 3 shows the *co tralateral* responses evoked by stimulation of muscle and skin nerves in the leg The ingoing volley from the muscle nerve was monitored by triphasic recording from the sciatic nerve (upper traces) the skin nerve was dissected too proximally to permit satisfactory recording of the ingoing volley Records A—E show discharges recorded from the dorsal fascicle (D) on stimulation of the muscle nerve at indicated strengths The initial part of the discharge was conspicuous at 1 2 times threshold (A) and maximal at 1 7

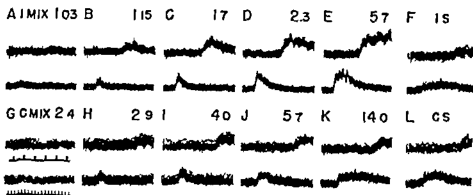


Fig 4 Discharges in ascending tracts recorded from dissected lateral funiculi on stimulation of ipsilateral (I) and contralateral (C) mixed radial (MIX) and skin (S) nerves in the wing The nerves of one side were stimulated and the effects of the volleys were investigated in the ipsilateral (A—F) and contralateral (G—L) funiculus Stimulus strengths for A—E and G—K indicated as multiples of the threshold for a barely visible discharge in the ipsilateral tract Upper and lower traces show the ascending discharges recorded simultaneously at different speeds Time scales in msec Distances stimulating electrode on mixed or skin nerve — spinal cord 8 0 cm spinal cord — recording site 13 cm

times threshold (C) Later components appeared at this strength and increased at higher strengths (D E) The initial part of the discharge was apparently due to a volley in low threshold muscle afferents which may correspond to the group I afferents in mammals At the temperature obtaining in our animals (about 38° C) the conduction velocity in the fastest muscle afferents was 75—85 m/sec in the various experiments (cf Fig 3 M—P) and the conduction velocity of the fastest fibres in the spinal cord 65—75 m/sec (cf Fig 3 Q) A synaptic elay of at most 0 7—1 0 msec can be calculated for the initial part of the ntralateral discharge evoked from low threshold muscle afferents This delay cludes the true synaptic delay and the delay due to the slow conduction velocity in the presynaptic branches it is similar to the values found for monosynaptic transmission in the mammalian cord It is concluded that the discharge was monosynaptically transmitted

Stimulation of the skin nerve evoked a slowly rising discharge without distinct front (Fig 3 F) The long latency suggests polysynaptic transmission

A small discharge (Fig 3 G—J) was evoked in the ventral fascicle (V) The records show that it was caused by impulses in afferents activated at 2 2 times threshold and higher strengths The latency is compatible with monosynaptic transmission when the slow conduction velocity in the primary afferents is taken into account Skin nerve stimulation produced a small but distinct monosynaptic response in the ventral fascicle (K L) The latency of the discharge was 0 4 msec shorter than that of the monosynaptic discharge evoked from the muscle nerve in the dorsal fascicle This difference is at least partly, due to the shorter conduction distance in the nerve (about 3 5 cm for the skin nerve and 7 cm for the muscle nerve)

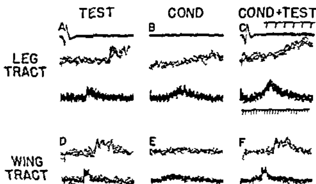


Fig 5 Inhibition of discharges evoked from low threshold muscle afferents in leg (A—C) and wing (D—F) nerves

Records A—C are from the experiment also shown in Fig 3 The lower traces show on a slow sweep the discharges evoked by the conditioning and testing stimuli and recorded from the dorsal half of the lateral funiculus The two upper traces in each vertical set of records were taken simultaneously and show on fast sweeps the ingoing volley recorded triphasically from the sciatic nerve (A C) and part of the lower trace respectively The test discharge (shown in isolation in A) was evoked by stimulation of the contralateral muscle nerve in the leg The conditioning volley was elicited in the contralateral skin nerve in the leg (the evoked ascending discharge is shown in isolation in B) Record C shows that the initial part of the test discharge was almost completely inhibited by the conditioning volley

Records D—F are from a different experiment Upper and lower traces correspond to middle and lower traces in A—C The test discharge (recorded from the dissected dorsal half of the lateral funiculus and shown in isolation in D) was evoked by stimulation of the ipsilateral radial nerve The conditioning volley was elicited in the ipsilateral skin nerve in the wing (the evoked ascending discharge is shown in isolation in E) Record F shows that the test discharge was little influenced by the conditioning volley The strength of the test stimuli was 2 5 times threshold and of the conditioning stimuli 10 times threshold (A—F) Time scales in msec

The large *ipsilateral* response (Fig 1 and 2 A) in the dorsomedial fascicle was investigated in some experiments On stimulation of muscle nerves it appeared when the strength had been raised above twice threshold Hence low threshold muscle afferents did not evoke any discharge in the dorsomedial fascicle neither did they evoke any ipsilateral discharge in more ventrally situated tracts

b "*Wing tracts* The discharges evoked from the wing nerves were analysed by separate stimulation of a mixed nerve and a cutaneous nerve The initial part of the ipsilateral discharge (Fig 1 and 2 B) appeared on stimulation of the mixed nerve but not on stimulation of the skin nerve (Fig 4) Simultaneous recording of the ascending volley in the dorsal funiculi (cf Methods) indicated that this initial part of the discharge was evoked by low threshold afferents the discharge appeared with a small volley and became maximal at a stimulus strength of approximately twice threshold The lack of any early discharge on stimulation of the skin nerve indicates that it was due to low threshold muscle afferents presumably corresponding to the group I afferents in mammals The "synaptic delay was calculated as 0 7—1 1 msec in the various experiments It indicates that the transmission was monosynaptic

[illegible] of the contralateral discharge evoked in fascicle (ii) [illegible] on stimulation of the mixed nerve as shown in Fig 4 [illegible] the discharges evoked by the same nerve were recorded in [illegible] (Fig 4 A—F) and the contralateral (G—K) dissected [illegible] The stimulus strengths given in the figure are multiples of [illegible] necessary for evoking a just perceptible ascending discharge in [illegible] fascicle (being only slightly above the threshold of the ingoing [illegible] above) The threshold of the contralateral discharge was [illegible] times the threshold for the ipsilateral discharge (compare G and A) [illegible] responsible primary afferents presumably had a conduction velocity [illegible] than 50 m/sec (cf values for the afferents in the muscle nerve Fig 3 [illegible]) The additional latency of the contralateral discharge relative to the [illegible] one varied in the different experiments from 0 8 to 1 3 msec This [illegible] latency is presumably accounted for by the longer conduction time [illegible] nerve Hence the latency suggests that the early part of the contralateral [illegible]harge was monosynaptically transmitted

Inhibition of discharges evoked from low threshold muscle afferents The discharge evoked from contralateral low threshold muscle afferents in leg nerves was [illegible]trongly inhibited by a conditioning volley in skin afferents or high threshold muscle afferents of the same limb In Fig 5 A the test discharge was evoked by stimulation of the muscle nerve at 2 5 times threshold only the initial part of the discharge was caused by a volley in low threshold muscle afferents (cf Fig 3 A—E) The conditioning volley in the skin nerve almost completely inhibited the initial part of the discharge (C) whereas the late part elicited by [illegible]es in high threshold afferents was little influenced

On the other hand the discharge evoked from low threshold muscle afferents in ipsilateral wing nerves was little influenced by a volley in cutaneous afferents or high threshold muscle afferents In Fig 5 D—F the effect of a conditioning volley in the skin nerve is shown The inhibition wa about 10 per cent at the optimal interval for inhibition Even a volley evoked by supramaximal stimulation of the radial nerve (which was also used for testing) resulted in an inhibition of only 10—20 per cent

Correlation The discharges in the avian cord cannot immediately be equated with any of the discharges previously observed in the mammalian cord The present discussion will be limited to a comparison of the discharges evoked from low threshold muscle afferents which have been investigated in detail in mammals

In mammals volleys in low threshold (group I) muscle afferents in hindlimb nerves evoke a discharge in the dorsal spinocerebellar tract (DSCT) on the ipsilateral side and in the ventral spinocerebellar tract (VSCT) on the contralateral side (OSCARSSON 1956 MAGNI and OSCARSSON 1962 a) In the bird low [illegible] muscle afferents in leg nerves did not produce any ipsilateral dis[illegible] This indicates either that the DSCT does not exist in the bird or that

it is not activated from low threshold muscle afferents On the other hand low threshold muscle afferents in leg nerves evoked a contralateral mono synaptic discharge which showed similarities with the VSCT discharge in mam mals It was due to activity in a tract located in the same area of the cord as the VSCT in mammals (HOLMQVIST and OSCARSSON 1963) which is also the area allocated to VSCT in the avian cord by WHITLOCK (1952 cf also KUHN and TRENDELENBURG 1911) The discharge was strongly inhibited by volleys in cutaneous and high threshold muscle afferents just as the VSCT discharge in mammals (OSCARSSON 1957 MAGNI and OSCARSSON 1962 a) Hence our re sults suggest that the VSCT exists in the bird and has a similar organization as in the mammal

In the cat group I muscle afferents in forelimb nerves activate only one ascending spinal tract (HOLMQVIST OSCARSSON and UDDENBERG 1963) This tract is uncrossed and not strongly inhibited from skin and high threshold muscle afferents (unpublished observations) its termination is unknown Similarly, in the bird there was only one spinal tract activated from low threshold muscle afferents in wing nerves This tract was uncrossed and little influenced from skin and high threshold muscle afferents Its location in the cord was more dorsal than the location of the group I activated forelimb tract in the cat Nevertheless the possibility that the two tracts are homologous should be con sidered

The anatomical descriptions of the spinocerebellar tracts in the bird are fragmentary and inconsistent FRIEDLANDER (1898) SANDERS (1929) KAPPERS HUBER and CROSBY (1936) LARSELL (1948) and WHITLOCK (1952) describe separate dorsal and ventral spinocerebellar tracts whereas SHIMAZONO (1912) and INGVAR (1918) do not separate the spinocerebellar system into two tracts Spinocerebellar tracts have been followed as far caudally as the lower lumbar region of the cord (FRIEDLANDER 1898 INGVAR 1912 WHITLOCK 1952)

Histological experiments to study the degree of crossing in the cord seem to have been performed only by KUHN and TRENDELENBURG (1911) They claim that the tract arising from lumbar segments is crossed at the spinal level and shifts gradually from a ventral position in the lower thoracic region to a dorsal position in the cervical region This dorsal position corresponds approximately to that described in this paper for the tract activated from low threshold muscle afferents in leg nerves On the other hand the spinocerebellar tract arising from the wing area was described as mainly uncrossed It was localized to the dorsal part of the lateral funiculus KUHN and TRENDELENBURG discussed the possibility that the crossed tract arising from the lumbar region is homol ogous to the VSCT and the uncrossed tract arising from the cervical cord to the DSCT

Conclusions

The present results show that coarse fibred ascending tracts have a similar organization in mammals and birds tracts in the dorsal part of the lateral funiculus are activated mono- and polysynaptically only from ipsilateral nerves whereas tracts in the ventral part of the lateral funiculus and in the ventral

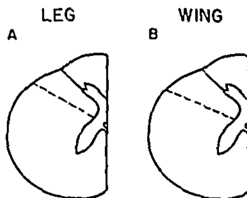


Fig 6 Cross sections of spinal cord at cervical level showing the areas occupied by uncrossed (dorsally of broken line) and crossed tracts (ventrally of this line) A shows the areas of tracts related to leg nerves and B the areas of tracts related to wing nerves

funiculus are monosynaptically activated only from contralateral nerves and polysynaptically, from both ipsilateral and contralateral nerves On analogy with the organization in the mammalian cord (MAGNI and OSCARSSON 1962 b HOLMQVIST and OSCARSSON 1963) it may be concluded that the former tracts originate from ipsilateral and the latter tracts from contralateral cell bodies i e *they are uncrossed and crossed tracts respectively*

In the cervical region the uncrossed tracts occupy the area dorsal of the interrupted line shown in Fig 6, A and B The area extends more ventrally for tracts activated from wing nerves (B) than for tracts activated from leg nerves (A) *In this respect there is also similarity between the mammalian and avian* cord, though the areas occupied by uncrossed tracts are smaller in the bird (cf Fig 6 in HOLMQVIST and OSCARSSON 1963)

Our observations give also information about some individual tracts in the duck It has previously been shown that the organization of the dorsal and ventral spinocerebellar tract (DSCT and VSCT) has been remarkably stable during the evolution of mammals These tracts are activated from group I muscle afferents in hindlimb nerves and have an almost identical organization in the investigated marsupial and placental species (MAGNI and OSCARSSON 1962 a) It has now been shown that the DSCT does not exist in the duck or, alternatively that it is not activated from low threshold muscle afferents On the other hand the duck has a tract activated from low threshold muscle afferents with similar characteristics as the VSCT in mammals *Presumably this* tract is identical with the VSCT but this can only be proved by demonstrating that it terminates in the cerebellum

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Organization of Ascending Tracts in the Spinal Cord of the Frog

By

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Abstract

Oscarsson O and I Rosén *Organization of ascending tracts in the spinal cord of the frog* Acta physiol scand 1963 *59* 154—160 — Discharges evoked in ascending spinal tracts on stimulation of the sciatic nerve were recorded from dissected fascicles of the cord As in mammals and birds dorsally located tracts are activated monosynaptically only from ipsilateral afferents whereas ventromedially located tracts are activated monosynaptically only from contralateral afferents It is concluded that the former tracts are uncrossed and the latter tracts crossed at the spinal level The overlapping of the areas containing uncrossed and crossed tracts is much larger in the amphibian than in the mammalian and avian cords The fastest fibres of the ascending tracts have a conduction velocity of about 10 m/sec

Electrophysiological investigations of coarse fibred ascending spinal tracts in mammals and birds have demonstrated that uncrossed and crossed tracts ascend in different areas of the cord The uncrossed tracts occupy the dorsal part of the lateral funiculus whereas the crossed tracts occupy the ventral part of this funiculus and the ventral funiculus (Magni and Oscarsson 1962 Holmqvist and Oscarsson 1963, Oscarsson Rosén and Uddenberg 1963) The main purpose of the present investigation was to determine if a similar pattern of organization exists in the amphibian cord

Methods

The experiments were made on frogs (*Rana temporaria*) anaesthetized by immersion in a 10 per cent urethane solution The frogs were washed in tap water immediately after the immersion which was stopped when the animals ceased moving The room temperature was 21—23 C

The sciatic nerve was dissected bilaterally and mounted on stimulating electrodes in pools of mineral oil The spinal cord was exposed by a laminectomy and usually transected rostrally of the third spinal roots (see diagram in Fig 1) Below the transsection the cord was split in the midline for a distance of 3—5 mm The dorsal funiculus was separated and mounted on electrodes for monophasic recording of the volley in primary afferents The remaining part of the cord half was also mounted on recording electrodes either undivided or after splitting into subdivisions of various sizes (called fascicles below) (cf LAPORTE LUNDBERG and OSCARSSON 1956) The incoming volley was routinely recorded triphasically with an electrode placed on the back of the frog at the level of the dorsal roots

The stimulating and recording technique has been described before (LAPORTE *et al* 1956 HOLMQVIST and OSCARSSON 1963) The stimulus strength is given as multiples of the nerve threshold The electrical events were displayed on two double beam oscilloscopes and recorded by photographic superposition The fascicles and lesions of the spinal cord were studied through a binocular preparation microscope after fixation in 10 per cent formalin

Results

The discharges evoked in ascending spinal tracts on stimulation of separately dissected muscle and skin nerves were too small to permit a detailed analysis Stimulation of the mixed sciatic nerve evoked relatively large responses in the dissected fascicles and has been used throughout this investigation In order to determine the nature of the synaptic linkage between primary afferents and tract neurones it was necessary to assess the conduction velocity of the primary afferents and tract fibres

1 Conduction velocities and synaptic delay

In the experiment of Fig 1 A the sciatic nerve was stimulated at about 5 times threshold and the primary afferent volley recorded from the dorsal funiculus the dorsal roots and the nerve dissected at successively more caudal levels The volley decreased in size at more rostral levels of the cord but was still visible in the rostral region of the cervical cord (cf record *c*) Presumably some of the primary afferents terminate in the dorsal funiculus nuclei as indicated by the experiments of BRAVO and DE MOLINA (1962) (cf KAPPERS HUBER and CROSBY 1936) In Fig 1 A the latency is plotted against distance from the stimulating electrode The latency at various levels of the cord can be derived from the inset diagram of the cord showing the intumescences dorsal root entries (dots) and entrance zone of roots contributing to the sciatic nerve (horizontal black bar) (cf GAUPP 1899) The conduction velocity of the fastest afferents was 28 m/sec in the nerve and dorsal roots and 9 m sec in the dorsal funiculus In 16 other experiments the velocity was calculated as 23—38 (mean 29) and 7—18 (mean 13) m sec respectively The threshold for evoking an afferent volley was the same when the recording was performed from the rostral part of the cervical cord as when it was performed from the dorsal roots It is concluded that the conduction velocity of individual fibres decreases shortly

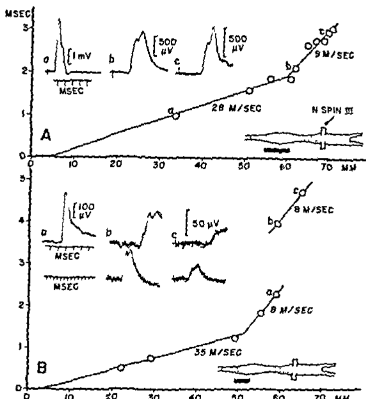


Fig 1 Conduction velocity in primary afferents and tract fibres. Synaptic delay.

A. The sciatic nerve was stimulated at a strength of 5 times threshold and the volley recorded from the dorsal funiculus, the dorsal roots, and the nerve dissected at successively more caudal levels. The latency is plotted against distance from stimulating electrode. Note spread of stimulus for a distance of some mm away from the electrode. The calculated conduction velocities are indicated. The diagram of the spinal cord shows the segmental levels at which recording was made. The dorsal root entries (dots) and the entrance zone of the roots contributing to the sciatic nerve (black bar) are indicated. The inset records correspond to the appropriately labelled points.

B. The discharge evoked in ascending spinal tracts by stimulation of the ipsilateral sciatic nerve at 5 times threshold was recorded at two segmental levels from the dissected cord half (except dorsal funiculus). The discharge was recorded at two speeds (upper and lower traces) and is shown by records b and c. The incoming volley was recorded as in A. Spinal cord diagram as in A, except that the interrupted part of the horizontal bar indicates the entrance zone of the small eighth root which presumably did not contribute to the sciatic nerve in this experiment. The temporal displacement of the presynaptic (a) and postsynaptic (b) response gives a synaptic delay of 1.7 msec.

after their entrance into the cord. In this respect there is similarity between the primary afferents of amphibians and mammals (cf. LLOYD and MCINTYRE 1950, REXED and STROM 1952).

The conduction velocity of the fastest tract fibres was determined by recording from the dissected "cord half" (except dorsal funiculus) at two different levels of the cord. *In Fig. 1 B the latency of the ipsilateral ascending discharge*

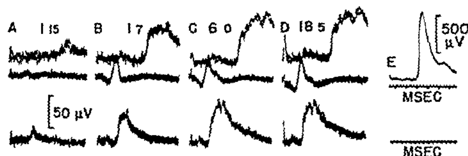


Fig 2 The discharge evoked in ascending spinal tracts on stimulation of the ipsilateral sciatic nerve The discharge was recorded from the dissected cord half (except dorsal funiculus) at a midthoracic level (A—D) Upper and lower traces show the discharge at different speeds the middle traces show the incoming volley recorded at the fast speed from the dorsal roots 15 mm more caudally Stimulus strength in multiples of nerve threshold is indicated Record E shows the volley in primary afferents recorded from the dorsal funiculus dissected at the same level as the remaining part of the cord half Note different voltage scales in A—D and E

(records b and c) was 4 75 and 4 00 msec respectively giving a conduction velocity of 8 m/sec In three other experiments values of 8 10 and 16 m/sec were obtained There was no consistent difference between the velocity of the fastest fibres contributing to the ipsilateral and contralateral discharge

As the primary afferent volley in the dorsal funiculus and the discharge in the secondary tract fibres are conducted at approximately the same velocity the synaptic delay is given by the temporal displacement of the presynaptic and postsynaptic responses recorded at the same segmental level In Fig 1 B and 2 the delay was about 1 7 msec In other experiments (20 cases) the delay varied between 1 4 and 2 6 (mean 1 9) msec and there was no consistent difference between the delay of the ipsilateral and contralateral response The delay introduced by a singly synapse in the amphibian central nervous system has been measured to about 1 5 msec for various synaptic regions (Eccles 1946 Fadiga and Brookhart 1960 Fukami 1961) It is concluded that the latency of the early part of the mass discharge does not allow for more than one synapse

2 Ascending discharges in the lateral and ventral funiculi

In Fig 2 A—D the upper and lower traces show the ascending discharge recorded at two speeds from the dissected cord half on stimulation of the ipsilateral sciatic nerve at indicated strengths The incoming volley (middle traces) was recorded triphasically from the dorsal roots 15 mm more caudally A small ingoing volley sufficed to evoke a discharge indicating a relatively efficient synaptic linkage (A) This was also demonstrated by the fact that the early part of the mass discharge did not decrease in size before the frequency of the stimulation had been raised above 50—100 per sec With increasing strength of stimulation there was a reduction in the latency and an increase in the amplitude of the early monosynaptic part of the discharge (B—D) There

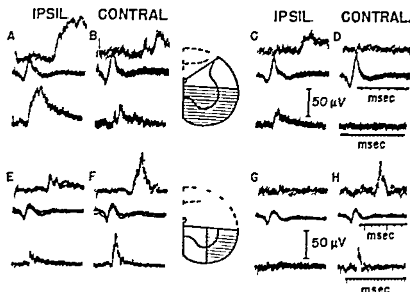


Fig 3 Distribution of ipsilateral and contralateral discharges in the lateral and ventral funiculi

A—D Discharges recorded from the dissected cord half (except dorsal funiculus see diagram) at mid thoracic level on stimulation of ipsilateral (A C) and contralateral (B D) sciatic nerve at 18 times threshold. Upper and lower traces show the ascending discharge at different speeds middle traces show the incoming volley recorded at the fast speed from the dorsal roots 15 mm more caudally A and B were obtained before and C and D after the lesion shown in the diagram (hatched) The voltage scale applies to the lower traces From the experiment also shown in Fig 2

E—H illustrate a different experiment. The records show discharges recorded from the dissected ventral quadrant of the cord (see diagram) at the upper thoracic level on stimulation of the *ipsilateral and contralateral* sciatic *nerve at 20 times threshold* A and F *were obtained be*fore and G and H after the lesion shown in the diagram (hatched) Conventions as in A—D

was also a marked increase in the duration of the mass discharge suggesting polysynaptic components Similar observations were made with the discharge evoked from the contralateral nerve

The discharge evoked by stimulation of the ipsilateral nerve was usually about twice as large as that evoked from the contralateral nerve (Fig 3 A and B) When the "cord half" was divided into one ventral and one dorsal part by horizontal splitting through the plane of the central canal the dorsal part gave *only an ipsilateral response and the ventral part* (Fig 3 E and F) both an ipsilateral and a contralateral response

In many experiments the distribution of the ipsilateral and contralateral discharges in the cord was studied by making lesions at the base of the dissected fascicle This method seemed to damage the preparation less than longitudinal splitting Fig 3 A—D shows records obtained from the "cord half" on stimulation of the ipsilateral and contralateral sciatic nerve The fascicle was originally intact and both ipsilateral and contralateral responses appeared (A and B) After a lesion in the ventral part of the lateral funiculus

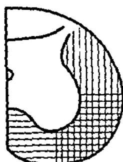


Fig 4 Spinal cord section through upper thoracic region showing the distribution of tracts activated monosynaptically from ipsilateral afferents (vertical hatching) and of tracts activated monosynaptically from contralateral afferents (horizontal hatching)

and the ventral funiculus (hatched in diagram) there remained only a reduced ipsilateral response (C and D) Records E and F were obtained from the ventral quadrant of the cord (see diagram) in a different experiment and show ipsilateral and contralateral responses The contralateral response was the larger one as in other experiments with recording from the ventral quadrant Only the contralateral discharge remained (G and H) after a lesion in the lateral part of the dissected fascicle (hatched in diagram)

A survey of the results obtained by recording from more than 40 variously dissected fascicles shows that the area containing fibres activated monosynaptically from ipsilateral nerves occupies the region indicated by vertical hatching in Fig 4 whereas the area containing fibres discharged monosynaptically from contralateral nerves occupies the horizontally hatched region

Discussion

It has recently been shown that uncrossed ascending spinal tracts in mammals and birds occupy the dorsal part of the lateral funiculus and crossed tracts the area ventral thereof (see Introduction) A similar organization of ascending tracts in the spinal cord of the frog is indicated by the present experiments In mammals primary afferents terminate almost exclusively on the ipsilateral side of the grey matter in the cord (for references see HOLMQVIST and OSCARSSON 1963) and this is also the case with the primary afferents in the frog (W W CHAMBERS and C N LIU personal communication) Accordingly a monosynaptic discharge evoked in ascending tracts from ipsilateral nerves may be taken to indicate that the tracts do not cross the midline and conversely a discharge evoked from contralateral nerves to indicate that they do cross the midline Hence our results indicate that the uncrossed tracts occupy the dorsal part of the cord and the crossed ones the ventral part The zone of overlapping is however large in the frog cord as shown by Fig 4 In contrast with the extensive overlapping of the areas containing uncrossed and crossed tracts in the frog there is little overlapping of these areas in mammals and birds when tracts from the same region for example the lumbar intumescence are considered (HOLMQVIST and OSCARSSON 1963 OSCARSSON *et al* 1963)

cell granules from the adrenal medulla and adrenergic nerves (SCHÜMANN 1960, EULER and LISHAJKO 1960)

In the heart treatment with reserpine depletes the tissue content of NA (BERTLER *et al* 1956 PAASONEN and KRAYER 1958, MUSCHOLL 1960 GAFFNEY *et al* 1962) and abolished the positive inotropic and chronotropic effects of tyramine (CARLSSON *et al* 1957, TRENDELENBURG *et al* 1961, CROUT *et al* 1962) Furthermore, tyramine has been shown to release NA from the heart *in vitro* and *in vivo* (BURN and BURN 1961, CHIDSEY *et al* 1962) However, there is still some debate of whether tyramine can deplete the cardiac stores of NA and whether the cardiac effects of this amine are entirely due to released NA. NASMYTH (1960) reported that the NA stores of guinea pig atria are not reduced significantly after exposure to tyramine He suggested that this amine has a direct effect and that the presence of NA plays a facilitatory role By contrast CHIDSEY *et al* (1962) reported a significant depletion of NA stores by tyramine in the dog atrium *in vivo* and concluded that NA release is directly involved in the cardiac actions of tyramine

In the studies reported herein the cardiac action of tyramine was re examined using isolated rabbit atria In these experiments the inotropic effects of tyramine were studied and catecholamine determinations were made in the same atria, thus providing a direct evaluation of the possible correlation of these two effects

Methods

Left atria from albino male rabbits were rapidly removed and placed in a specially constructed tissue bath perfused continuously with a modified Locke s solution at a rate of 12—14 ml/min and aerated with carbogen All preparations were stretched to an initial tension of 2 g and were driven at 90 beats/min with a Grass S4 stimulator Both electrical and mechanical responses were recorded on a Grass oscillograph For the former a Tetronix 122 amplifier was used The latter were recorded through the use of a force displacement transducer (Grass FT 03 or Sanborn SFX) Details of the preparation and instrumentation have been reported (TORCHIANA and ANGELAKOS 1963)

Tyramine was added to the perfusion fluid and the solution was freshly made every 30 min It was established that under the experimental conditions used the concentration of tyramine did not decrease more than 10 per cent over a period of one hour For this the concentration of tyramine before and after perfusion through the tissue was repeatedly determined and compared to freshly made solutions using the self fluorescence of this compound measured with an Aminco spectrofluorimeter

For the NA analyses the tissue was blotted dry weighed and minced in 10 per cent trichloracetic acid After filtering the NA content was determined according to the method of EULER and LISHAJKO (1961) Briefly this method involves adsorption on alumina at pH 8 5 elution with acetic acid and oxidation at pH 6 5 with potassium ferricyanide Recoveries range between 70—80 per cent The values reported were not corrected for recovery It is estimated that due to the small amount of tissue involved the reported values are known with a precision of approximately 0 05 μg g Measurements were made in a Coleman fluorimeter with both filters A (395/490 mμ) and B (435/540 mμ) On several occasions the samples were also measured on the Aminco spectrofluorimeter Only the NA values are reported since no measurable amounts of

Table I Contractile tension and NA content of atria perfused with tyramine

Group	Treatment	N of atria	NA (μg/g)		NA in treated as % of controls	T as % of peak response to tyramine
			Controls	Treated		
I	In Locke's solution for 5—6 hrs	8	1.50 ± 0.19	1.58 ± 0.34	105	—
	Tyramine (100 μg/ml)					
II	30 min	5	1.26 ± 0.20	1.16 ± 0.29	92	6
III	90—180 min	8	1.37 ± 0.13	0.89 ± 0.26	74	48
	Subgroup A	3	1.12 ± 0.20	1.23 ± 0.25	110	68
	Subgroup B	5	1.38 ± 0.28	0.68 ± 0.18	49	36
IV	At the time when unresponsive to tyramine (3—6 hrs)	4	1.91 ± 0.24	0.87 ± 0.37	46	0
V	30 min after the end of tyramine infusion	4	1.51 ± 0.20	1.00 ± 0.26	66	4*

*Average effect when challenged with tyramine after washing for 30 min.

adrenaline (A) were found. Later measurements (ANGELAKOS and TORCHIANA 1963) with atria pooled from several rabbits indicated that the A content of the left atrium was less than 0.01 μg/g.

Residual amounts of tyramine that may be left in the tissue do not interfere with determinations of NA since this amine does not adsorb on alumina (EULER 1956). Furthermore it was found experimentally that the presence of as much as 100 μg of tyramine does not interfere with the determination of 1 μg of NA with the fluorimetric method used.

Results

In the early part of these studies distinct differences were observed in the atrial NA content of groups of animals received at different times. In general smaller male animals (2—3 kg) had a higher NA content than larger rabbits (4—5 kg) of the same sex. By contrast the atrial NA content within a given group was fairly uniform. Thus it was found necessary to run parallel controls from each group of animals. In each case the NA content for each experimental group was compared to that of concurrently run controls (Table I).

It is pertinent to note that the NA content of controls did not change even after perfusion with modified Locke's solution for a period of 5—6 hours. No significant changes in the isometric tension were observed for a period up to 4 hours although a moderate decrease in tension occurred in some preparations after 4—5 hours.

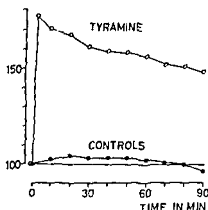


Fig 1 Average tension of 5 atria during continuous perfusion with tyramine (100 μg/ml)

Perfusion with tyramine in concentrations of 10—100 μg/ml produced a consistent positive inotropic effect It is noteworthy that this response was obtained with the atrium driven at a *constant rate and therefore is not complicated* by concurrent changes in the pacemaker rate In preliminary experiments concentrations of 20 μg/ml produced in two atria an average increase in contractility of 27 per cent In the next 5 experiments 100 μg/ml increased contractility by an average of 64 per cent while 200 μg/ml in 2 experiments produced an average increase of 94 per cent On the basis of these studies the concentration of 100 μg/ml was chosen for most of the subsequent experiments since it was desirable to study the response of the preparation when the effect was definite and nearly maximal With this concentration the average increase *in contractile force in a total of 20 atria* was 72 per cent In absolute units this corresponds to an increase of 0 5—1 5 g tension

A similar increase in contractility as that following 100 μg/ml of tyramine was obtained with 0 5—1 0 μg/ml of NA Dichloroisoproterenol (5 μg/ml) was somewhat more effective in blocking the effects of these equipotent concentrations of tyramine than of NA

As anticipated from the results of previous studies (NASMYTH 1960 CHIDSEY *et al* 1962) during the continuous perfusion with tyramine there was a gradual decrease in the positive inotropic response even though the concentration of the amine was maintained constant However it was found that the response decreased very slowly over several hours and the time course was not uniformly consistant from atrium to atrium

The average tension of 5 atria exposed to tyramine for 90 min is shown in Fig 1 It is clear that at the end of this period the tissue is still responding to tyramine and there is only a moderate reduction of the peak effect In other experiments perfusion was continued for 3—6 hours in order to obtain a substantial reduction of the tissue response to tyramine In still another group of ex

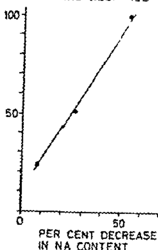


Fig 2 Correlation between reduction in positive inotropic response and NA concentration during continuous perfusion with tyramine

periments the perfusion was terminated at the end of 30 min The results are summarized in Table I The NA of the tissues determined at the end of the experiment is also included in the table Only NA values are reported since there were no measurable amounts of A in these tissues It is of interest that atria perfused for 90 min or more could be subdivided into two groups A group (III A) in which the response to tyramine was still 65 per cent or more of peak when the perfusion was terminated and another group (III B) in which the response was reduced to 50 per cent or less of peak Group IV consists of separate group of four atria in which tyramine was perfused until the response was completely abolished

The corresponding NA concentrations shown in the table for each of these groups are of particular interest The differences between the controls and the 30 min perfusion (group II) with tyramine is not statistically significant However following exposure for 90—180 min (group III) the difference is quite definite ($p = < 0.05$) and becomes quite clear when the subgroups III A and III B are compared Fig 2 shows the relationship between the reduction in NA stores and in the tyramine response

To test the ability of the tissue to restore its NA content atria were perfused with tyramine until the positive inotropic response had virtually disappeared (less than 5 per cent of peak) and were subsequently washed for 30 min At the end of this period the response to tyramine (100 μg ml) was tested briefly (10 min) In most cases no significant response was observed following this second exposure to tyramine At the end of this period the tissues were reserved for NA analysis The results are included in Table I (group V)

In similar experiments the ability of the tissue to respond to added NA was

tested after the inotropic response to tyramine had disappeared. The tissue was found to respond to the same extent as before the tyramine treatment. Such brief exposure to NA was found to restore partially the response to tyramine. In other experiments it was found that exposure to test doses of NA in the process of obtaining data for a dose response curve increased markedly the NA content of the tissue analyzed after washing for one hour following the last NA dose. Thus 5 atria tested in this manner with doses ranging from 0.1 to 8.0 μg/ml had an average NA content of 2.93 μg/g. The value for the corresponding controls was 1.15 μg/g.

In another series of 11 exp. left atria were exposed repeatedly to tyramine concentrations of 20—100 μ/ml and were subsequently washed for periods of 1 hour or more. In all cases it was observed that the contractility returned eventually to control levels. There was no evidence that contractility was depressed *following tyramine even when the atria were exposed to large doses (100 μg/ml)* for prolonged periods (90 min to 6 hours). On the contrary, it was observed that during washing following exposure to tyramine there was a transient increase in contractile force. This was of the order of 10 per cent lasting for 2—3 min. It was more marked following a short rather than a long exposure to tyramine.

Discussion

It has been shown that when the cardiac NA stores are depleted by pretreatment with reserpine the positive inotropic response to tyramine is abolished (Crout *et al.* 1962, Muscholl 1960). In the present experiments the reverse approach was used and the catecholamine content of tissues was examined when the response to tyramine was reduced or abolished. The overall results indicate that when the tyramine response was reduced substantially the NA content was significantly less than in the controls. Although a strict correlation could not be demonstrated it is clear that a progressive decrease in the tyramine response is associated with lower NA content. In this connection it is noteworthy that the response is greatly reduced or completely abolished even though the tissue content of NA is still approximately 50—40 per cent of normal. This could be interpreted as indicating that not all NA stores are readily available for release with tyramine. Crout *et al.* (1962) using reserpinized animals in various stages of NA depletion found that the response of the guinea pig atria to tyramine was not affected until the tissue content was reduced to more than 50 per cent of the control. If the responses of the guinea pig atria and rabbit atria are comparable the results of these authors can be brought to agreement with the present observations by suggesting that small doses of reserpine and *tyramine have a different mode of action* or act to release different fractions of the NA stores. This is essentially the same as the overall conclusions of Crout *et al.* (1962) based on the assumption that the NA stores are contained into two

compartments (TRENDELENBURG 1961) However there are still certain quantitative differences which can not be fully accounted for In general depletion of NA stores by tyramine is limited and apparently different from that produced by reserpine This is in agreement with observations on isolated nerve granules (EULER and LISHAJKO 1960 1961)

The present results on rabbit atria contrast with those reported by NASMYTH (1960) in guinea pig hearts He found no reduction in the NA stores after exposure to tyramine However the doses employed were smaller and the time of exposure was shorter in Nasmyth s experiments than in the present studies It is clear from the present results that exposure of the rabbit atria to even high concentrations of tyramine for 30 min does not produce a significant decrease in the NA content However over the same period the positive inotropic response to tyramine was reduced to less than 25 per cent Similarly it is possible that smaller doses of tyramine do not produce a detectable decrease in the NA stores It is clear that the present results do not provide any direct proof that tyramine acts by releasing NA from tissue stores as suggested by BURN and RAND (1958) although they are in agreement with that hypothesis However the alternative suggestion that (NASMYTH 1960 KUSCHINSKY *et al* 1960) the presence of NA plays a permissive role to a direct action of tyramine is equally tenable

The persistence of the tyramine response over several hours is striking It is rather unlikely that the total amount of NA present in a tissue of approximately 0 2 g (NA ca 0 3 μg) is sufficient to maintain the tissue contractility at 50—100 per cent above control levels for a period of 2—3 hours For a similar response with administered NA the final concentration needed is 0 5—1 0 μg/ml This is particularly striking considering the fact that the tissue was perfused at a rate of 14 ml/min and the maximum depletion observed was of the order of 50 per cent or a total amount of approximately 0 15 μg of NA This coupled with the observation that the NA depletion is slow may be taken to suggest that resynthesis of NA proceeds fairly rapidly even in the isolated atrium *in vitro* It is evident from the results (group V) that 30 min after depletion with tyramine the tissue synthesized approximately 20 per cent of the average control value However this increase did not restore the response to tyramine This could be interpreted as indicating that the newly synthesized NA was associated with a fraction which was not readily available for tyramine release

It was of particular interest to note that following maximal depletion of the NA stores by tyramine and subsequent washing the contractility of the atria is still normal This indicates that there is no direct correlation between catecholamine stores and myocardial contractility This is in agreement with the observations of KAKO *et al* (1960) that the spontaneous catecholamine depletion observed in the isolated perfused dog heart could not be correlated with myocardial efficiency However it is possible that such a correlation becomes significant when the catecholamine levels are exceedingly low as suggested by LEE and SHIDEMAN (1959) on the basis of experiments on reserpinized animals

The authors wish to acknowledge the encouragement and guidance given by Professor U S von Euler throughout the course of these studies and the expert advice of Engineer F Lishajko in the determination of tissue catecholamines is gratefully acknowledged

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Acta physiol scand 1963 59 169—183

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Gastric Acid Secretion in Gastric Fistula Cats during Reserpine Treatment[1]

By

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Abstract

EMÅS S *Gastric Acid secretion in gastric fistula cats during reserpine treatment* Acta physiol scand 1963 *59* 169—183 —The effect of daily intramuscular injections of reserpine on basal gastric secretion and on the secretory responses to intravenous histamine gastrin metha choline and insulin hypoglycemia was investigated in nonanesthetized gastric fistula cats Reserpine treatment during 3 days or more produced an elevation of basal secretion and the secretory responses to histamine and gastrin were increased while that to methacholine re mained unaltered and that to insulin hypoglycemia was reduced The changes described were highly significant The results suggest at least a two fold mode of action of reserpine on the gastric sec etory mecha nism The experiments with histamine and gastrin indicate that reserpine treatment increases the excitability of the parietal cells to these stimuli those with methacholine and insul n hypoglycemia indicate that reserpine treatment also interferes with the gastrin mechanism

In 1954 PLUMMER *et al* and BARRETT RUTLEDGE and ROGIE reported that a single intravenous injection of reserpine stimulated gastric secretion of acid Several papers dealing with the secretagogue action of reserpine have since appeared (for references see GATI *et al* 1960)

The effect of prolonged reserpine treatment on basal gastric secretion has been studied in human beings In investigations with daily reserpine doses

[1] A preliminary repo t of this nvest gation was p esented at the Tenth Scand navian Physiological Congress n O lo Norway 1960 (EMÅS 1960 a)

of 0 75 to 1 mg no appreciable change in basal secretion has been observed (RIDER 1955, DRENICK 1956 WOLF and ROSSMAN 1956 KIRSNER and FORD 1957 SCHNEIDER and CLARK 1957), and even following daily doses of 15 mg gastric secretion within the average range has been reported in two patients (BACHRACH 1959) Increases of gastric acid secretion have been reported on the other hand following daily reserpine doses of 0 32 and 0 5 mg (LIEBOWITZ and CARBONE 1957) and 2 to 3 mg (HAVERBACK *et al* 1955) In the last mentioned investigation the secretory studies started 3 hours after reserpine had been given It is well established that the secretagogue action of a single dose of reserpine lasts for several hours The observed increase *therefore represents* not only the possible effect of prolonged reserpine treatment but also the secretagogue action of the last reserpine dose

No experimental data are available concerning the effect on basal gastric secretion of prolonged reserpine treatment with doses of reserpine larger than those used clinically Nor does the literature contain any studies on the effect of reserpine treatment on gastric secretion elicited by different stimuli Such studies seem worthwhile in that such treatment may exercise an influence on factors that are conceivably of importance for the regulation of gastric secretion (discussed later in this paper)

The present study on nonanesthetized gastric fistula cats deals with the effect of prolonged reserpine treatment upon basal secretion and upon the secretory responses to histamine gastrin methacholine and insulin hypoglycemia

Methods

The experiments were performed on 9 nonanesthetized gastric fistula cats (EMÅS 1960 b) covering both sexes and weighing 3 0 to 4 1 kg

The basal gastric secretion and the secretory responses to histamine gastrin methacholine (Betacholyl©) and or insulin hypoglycemia were determined in each animal (controls The animals were then subjected to daily reserpine (Serpedin©) injections and the experiments were repeated

Experimental Technique

The experiment started in the morning after the animals had starved for 18 to 20 hours. Basal secretion was recorded for at least one hour before any secretory excitant was administered Gastrin dissolved in acidified physiological saline (JORPES JALLING and MUTT 1952) or histamine or methacholine dissolved in physiological saline was then infused i v for a 15-min period during each of 4 hours by means of an infusion pump (infusion rate 0 40 ml per min Insulin was administered as a single i injection. To determine the blood sugar level two samples of venous blood were collected before the injection of insulin and then every half hour for two hours The samples were analyzed by the anthrone method DURHAM *et al* 1950

The 15-min gastric secretion was recorded and the amount of acid determined by titration against 0 01 N NaOH Topfer s reagent indicated the endpoint for the titration of free acid and phenolphthalein that for total acid The amount of acid secreted was expressed in milliequivalents meq Reserpinization produced increased basal secre

tion which was therefore corrected for in all experiments The secretory re p nses to histamine gastrin and methacholine are accordingly defined as meq of total acid se creted during 1 hour from the commencement of infusion (EMAS 1960 b) minus the 1 hour basal secretion, and that to insulin as meq of total acid secreted during 3 hours following injection minus 3 times the 1 hour basal secretion

The doses of histamine (as the dihydrochloride) and gastrin infused elicited in the control experiments secretory responses of less than 50 per cent of the maximal secretory capacity of the cat s stomach (EMÅS unpublished observations) In the experiments with methacholine there were chosen thoses doses (as the iodide) that produced acid secretory responses with as small effects as possible on salivation The secretory responses to in sulin hypoglycemia were submaximal (EMÅS unpublished observations) and in most control experiments the blood sugar level lay during maximal hypoglycemia between 40 and 60 mg glucose per 100 ml blood All the doses given in the tables and figures are related to the animal s weight at the beginning of this series The total dose was then kept constant throughout the series despite a weight loss of 5 to 15 per cent in most animals during the periods of reserpinization

The two gastrin preparations used (3p and 9p) were prepared from antral mucosa of hogs by the method of JORPES *et al* (1952) in a somewhat shortened version (EMAS 1960 b) The secretory activity per mg of preparation 3p and 9p amounted to 50 and 100 histamine units (UVNAS and EMAS 1961) respectively No histamine that could be responsible for the secretory effects was found in the preparations when assayed on guinea pig ileum (<0.1 μg of histamine dihydrochloride per mg)

Reserpine Treatment

For a period of usually 7 to 13 days (mean 11 days) reserpine was administered i m in a daily dose of 0 10 to 0 15 mg per kg of body weight at the same time each day After 3 days treatment with reserpine — the animal is then referred to as reserpinized — the experiments described above were repeated every 3rd or 4th day On the days of ex periment reserpine was not injected until after the experiment so that when the expe riment started 20 to 24 hours had passed since the last injection

The definition of reserpinized cats was based on the observation (made in preliminary experiments) that at least 3 days of reserpine treatment was required to produce regularly the increase in responses to histamine and gastrin that is to be described

Symptoms frequently appearing during reserpinization were miosis relaxation of the nictitating membrane diarrhea and marked sedation As a rule the animals did not eat by themselves and had to be fed twice a day by hand Reserpinization was discontin ued if there were obvious risks of the animal succombing Up to 6 periods of reserpiniza tion were effected on the same individual The intervals between these periods varied from 2 weeks to 4 months but were in most cases of 4 weeks or more Different secre tory stimuli were tested within the same period Histamine or gastrin however was included in all periods

Evaluation of Data

The variance for the basal sec etion of the individual animal differs considerably as between the nonreserpinized and reserpinized state and the rank sum test (DIXON and MASSEY 1957) was therefore used in the analysis of the experimental data

In general the variance for the secretory esponses of the reserpinized animal is also larger than that of the same but nonreserpinized animal In view however of the rela tively small number of experiments per individual with each secretory stimulus the experimental data were processed by analysis of variance Since it has been impossible in the present study to maintain symmetry ove the series a modification of the ordinary

Table I Mean 1-hour basal secretion and mean 1 hour secretory responses to 4 repeated intravenous infusions of histamine in gastric fistula cats nonreserpinized and reserpinized

Cat no	Histamine in mg/kg	Nonreserpinized (controls)						Reserpinized						Increase of mean response to 1st infusions during reserpinization	
		N of exp	Secretion in meq total acid					N of exp	Secretion in meq total acid					In meq total acid	In per cent of mean for controls
			Basal secretion	Response[1] to histamine infusion no					Basal secretion	Response to histamine infusion no					
				1	2	3	4			1	2	3	4		
39	0.010	6	0.01	**0.48** [2]0.36– 0.62	0.49	0.45	0.43	4	0.09	**0.80** 0.53– 1.08	0.69	0.63	0.67	**0.32**	67
40	0.010	4	0.03	**0.34** 0.24– 0.44	0.37	0.30	0.33	2	0.09	**0.79** 0.78– 0.79	0.88	0.65	0.61	**0.45**	132
41	0.010	5	0.04	**0.46** 0.41– 0.55	0.51	0.56	0.51	5	0.17	**1.25** 0.96– 1.68	1.16	1.06	0.98	**0.79**	172
45	0.015	4	0.05	**0.28** 0.20– 0.39	0.28	0.25	0.26	5	0.34	**1.04** 0.64– 1.59	0.92	0.48	0.45	**0.76**	271
48	0.010	6	0.05	**0.23** 0.15– 0.28	0.25	0.26	0.25	4	0.20	**0.77** 0.54– 1.12	0.86	0.74	0.64	**0.54**	235
71	0.015	4	0.07	**0.21** 0.13– 0.28	0.21	0.16	0.18	4	0.12	**0.56** 0.43– 0.79	0.54	0.54	0.48	**0.35**	167
74	0.010	5	0.02	**0.24** 0.15– 0.31	0.24	0.20	0.25	4	0.15	**0.52** 0.37– 0.69	0.59	0.56	0.50	**0.28**	117
78	0.015	4	0.01	**0.18** 0.13– 0.22	0.18	0.18	0.16	3	0.05	**0.54** 0.51– 0.57	0.55	0.53	0.41	**0.36**	200

[1] Correction made for basal secretion.
[2] Range of secretory responses.
Illustrated in Fig 1

procedure of analysis of variance has been necessary (SNEDECOR 1956 Chapt 12 Sections 14 and 15) It was found that the difference between the above mentioned variances was not so large as to invalidate the analysis

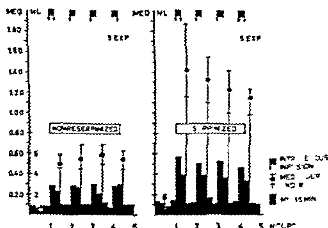


Fig 3 Basal gastric secretion and secretory responses (uncorrected) to repeated i v infusions of histamine in a gastric fistula cat (no 41) nonreserpinized (mean of 5 exp) and reserpinized (mean of 5 exp) Dose of histamine 0 010 mg per kg of body weight for 15 min.

Results

Basal Secretion (Table I to IV)

In nonreserpinized cats (105 experiments) basal secretion only rarely contained titratable amounts of free acid and the mean 1 hour basal secretion of total acid varied between less than 0 01 meq (cat 78) and 0 05 meq (cat 45)

During reserpinization (87 exp) free acid was observed in most experiments and the mean basal secretion of total acid was elevated in all animals The mean 1 hour output of total acid of each individual fell within the range of 0 07 meq (cat 78) to 0 42 meq (cat 45) The mean increase for individual cats varied between 0 06 meq (cat 40) and 0 37 meq (cat 45) and a large increase appeared to be related to a comparatively high basal secretion in the nonreserpinized state The rank sum test (DIXON and MASSEY 1957) showed that the basal acid secretion of the animal nonreserpinized and reserpinized differed highly significantly ($P < 0\ 001$) in 7 of 9 cats studied In the remaining 2 cats (no 71 and 74) the number of experiments was small and basal secretion differed almost significantly ($P < 0\ 05$)

In 12 exp on reserpinized cats basal acid secretion was determined also *after* the repeated infusions of secretory excitant and was found still to be elevated justifying the definition of secretory response

Secretory Responses to Histamine (Table I)

In 8 nonreserpinized cats investigated repeated infusions of histamine produced acid secretory responses that were fairly uniform both within the same experiment and on the same animal from one experiment to another Results corresponding in principle to those obtained here have been statistically analyzed elsewhere (EMÅS 1960 b)

Table II Mean 1 hour basal secretion and mean 1 hour secretory responses to 4 repeated intravenous infusions of gastrin in gastric fistula cats nonreserpinized and reserpinized

Cat no	Gastrin prep no	Gastrin in mg/kg	Nonreserpinized (controls)						Reserpinized						Increase of mean response to 1st infusions during reserpinization	
			N of exp	Secretion in meq total acid					N of exp	Secretion in meq total acid					In meq total acid	In per cent of mean for controls
				Basal secretion	Response[1] to gastrin infusion no					Basal secretion	Response to gastrin infusion no					
					1	2	3	4			1	2	3	4		
39	3p	0.25	4	0.01	**0.46** 0.41–0.58	0.42	0.35	0.33	4	0.14	**0.72** 0.51–0.94	0.77	0.69	0.60	**0.26**	57
40	3p	0.35	2	0.03	**0.40** 0.40–0.40	0.44	0.39	0.38	2	0.10	**0.99** 0.90–1.07	1.12	0.77	0.58	**0.59**	148
[2]41	3p	0.35	3	0.02	**0.57** 0.54–0.60	0.62	0.54	0.46	3	0.30	**0.99** 0.76–1.13	0.82	0.55	0.41	**0.42**	74
[2]45	3p	0.35	3	0.05	**0.21** 0.17–0.29	0.17	0.17	0.19	3	0.34	**0.78** 0.44–1.18	0.56	0.24	0.20	**0.57**	271
[2]48	3p	0.35	3	0.02	**0.26** 0.23–0.29	0.34	0.23	0.21	4	0.22	**0.97** 0.58–1.27	1.06	0.88	0.67	**0.71**	273
110	9p	0.20	5	0.01	**0.42** 0.36–0.52	0.52	0.47	0.43	4	0.08	**0.74** 0.64–0.97	0.71	0.64	0.60	**0.32**	76

Correction made for basal secretion.
[2] Individual experiments illustrated in a previous paper (Emås 1960 b Fig 8)
[3] Range of secretory responses
Illustrated in Fig 2

Reserpinization produced increased secretory responses to histamine in all 8 animals In agreement with earlier findings (Emås 1960 b) initially large secretory responses declined with repeated stimulation The mean responses of cat 41 nonreserpinized and reserpinized are illustrated in Fig 1 Owing to this decline only the response to the first infusion in each experiment has been used when evaluating the effect of reserpinization For individual cats the mean increase ranged from 0 28 meq of total acid (cat 74) to 0 79 meq (cat 41)

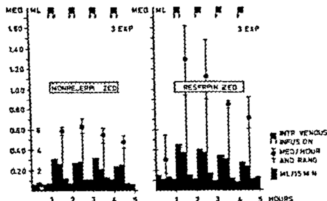


Fig 2 Basal gastric secretion and secretory responses (uncorrected) to repeated i v infusions of gastrin in a gastric fistula cat (no 41) nonreserpinized (mean of 3 exp) and reserpinized (mean of 3 exp) Dose of the gastrin preparation 0 35 mg per kg of body weight for 15 min The acid output in each experiment on the nonreserpinized animal has been illustrated elsewhere (EMAS 1960 b Fig 8)

and the percentage increase from 67 (cat 39) to 271 (cat 4ↄ) The difference between the acid secretory responses of nonreserpinized and reserpinized cats was highly significant ($P < 0\ 001$) according to the analysis of variance (SNEDECOR 1956) When reserpine treatment was discontinued the effect on the secretory responses persisted for different lengths of time with different individuals but the responses of most animals were normal after 4 days

Secretory Responses to Gastrin (Table II)

Of 6 cats tested 5 were used in the above mentioned experiments with histamine The results obtained with gastrin were similar in principle to those obtained with histamine

In the nonreserpinized cats fairly uniform responses to gastrin were obtained in the same animal both within experiments and from one experiment to another Three experiments on each of cats no 39 41 45 and 48 have been illustrated and statistically analyzed elsewhere (EMAS 1960 b)

Reserpinization increased the mean secretory responses to gastrin in all 6 cats With hourly repeated stimulation there was an obvious decline in the large secretory responses Fig 2 demonstrates the effect of reserpinization on the mean secretory response of cat 41 Reserpinization increased the mean secretory response to the first gastrin infusions by 0 26 meq of total acid or ↄ7 per cent (cat 39) to 0 71 meq or 273 per cent (cat 48) Analysis of variance demonstrated a highly significant ($P < 0\ 001$) difference between the acid secretory responses of the nonreserpinized and reserpinized cats

Secretory Responses to Methacholine (Table III)

All 6 cats tested were included in the experiments with histamine and/or gastrin

In the nonreserpinized cats the variation expressed in meq total acid of the first secretory responses in experiments on the same individual was com

Table III Mean 1 hour basal secretion and mean 1 hour secretory responses to 4 repeated intravenous infusions of methacholine in gastric fistula cats nonreserpinized and reserpinized

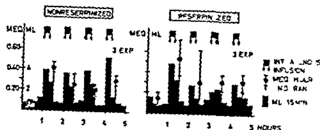
Cat no	Methacholine in mg/kg	Nonreserpinized (controls)						Reserpinized						Increase of mean response to 1st infusions during reserpinization	
		N of exp	Secretion in meq total acid					N of exp	Secretion in meq total acid					In meq total acid	In per cent of mean for controls
			Basal secretion	Response to methacholine infusion no					Basal secretion	Response[1] to methacholine infusion no					
				1	2	3	4			1	2	3	4		
39	0 020	3	0 04	**0 21** 0 16–0 32	0.23	0 12	0 14	3	0 05	**0 17** 0 07–0 31	0 07	0 09	0 04	**-0 04**	(-19)
*41	0 020	3	0 04	**0.36** 0.33–0 42	0.21	0 15	0 25	3	0 15	**0.38** 0.29–0 52	0 16	0 16	0 17	**0 02**	(6)
45	0 020	4	0 04	**0 07** 0 04–0 11	0 05	0 04	0 03	4	0 61	**0 14** 0 03–0 35	0 11	0 16	0 00	**0 07**	(100)
48	0 015	3	0 02	**0 11** 0 06–0 15	0 07	0 13	0 12	3	0 15	**0 17** 0 11–0.22	0 13	0 16	0 17	**0 06**	(55)
78	0 040	4	0 00	**0 12** 0 08–0 14	0 12	0 12	0 11	2	0 03	**0.26** 0 11–0 40	0 20	0 13	0 12	**0 14**	(117)
110	0 030	4	0 03	**0.20** 0 16–0.29	0 17	0 15	0 14	3	0 10	**0 08** 0 04–0 12	0 02	0 01	0 01	**-0 12**	(-60)

Correction made for basal secretion.
* Range of secretory responses.
* Illustrated in Fig 3

parable to that of the responses to histamine and gastrin Within several individual experiments however the secretory responses tended to decline with repeated stimulation The significance of this decline was not analyzed statistically as only the first response in each experiment was used in evaluating the effect of reserpinization

The reserpinization of the cats produced no very marked changes in secretory response The mean response to the first infusions increased in 4 cats by between 0 02 meq (cat 41) and 0 14 meq (cat 78) while in 2 cats it decreased

Fig 3 Basal gastric secretion and secretory responses (uncorrected) to repeated i v infusions of methacholine in a gastric fistula cat (no 41) nonreserpinized (mean of 3 exp) and reserpinized (mean of 3 exp) Dose of metacholine 0 020 mg per kg of body weight for 15 min



by 0 04 meq (cat 39) and 0 12 meq (cat 110) The secretory responses of cat 45 during reserpinization are unreliable owing to the large basal secretion Analysis of variance revealed no significant ($P > 0.05$) difference between the responses of nonreserpinized and reserpinized cats The small secretory responses resulting from the author s concern to keep down salivation mean that the figures for percentage change might be misleading Fig 3 shows the mean responses of cat 41 nonreserpinized and reserpinized

The infusions of methacholine elicited salivation and it was possible that the admixture of saliva to the gastric juice might have reduced the figures for the output of total acid This was investigated by adding saliva — collected from 2 anesthetized cats (preparation see EMAS 1960 b) continuously infused with methacholine — to acid gastric juice obtained from nonanesthetized gastric fistula cats continuously infused with histamine In two series 1 2 3 and 4 ml of saliva were added to 2 ml of gastric juice and total acid determined (5 titrations on each sample) With increasing amounts of saliva the titratable amounts of total acid were reduced linearly and extrapolation revealed that 6 and 9 ml of saliva respectively would have to be added to reduce the amounts of total acid from 0 23 and 0 29 meq to zero

The main problem, however was whether methacholine elicits an increased flow of saliva during reserpinization, thereby masking increased responses of total acid If this were the case methacholine would elicit a greater volume of gastric juice in the animal when reserpinized than when nonreserpinized In fact the corrected mean volume of gastric juice following the first methacholine infusions decreased during reserpinization in 4 cats by 0 9 ml (cat 39) to 5 0 ml (cat 45) and increased in 2 cats by only 1.2 ml (cat 41) and 0 8 ml (cat 48)

Secretory Responses to Insulin Hypoglycemia (Table II)

All 6 cats tested were used in the experiments with histamine and 5 of them also in those with gastrin and methacholine

In the nonreserpinized cats the secretory response appeared within one hour after the injection of insulin After a further hour acid secretion had returned to its preinjection level in most experiments and at the end of the 3rd post injection hour in all experiments

Reserpinization reduced the mean secretory response in all animals Fig 4 shows the mean responses of cat 41 The secretory response during reserpinization exceeded the minimum response of the same but nonreserpinized animal in only 2 exp (on cat 39) The reduction of the mean respons- — · · -

Table II Mean 1 hour basal secretion and mean 3 hour secretory response to insulin hypoglycemia in gastric fistula cats nonreserpinized and reserpinized

Cat no	Insulin in IU/kg	Nonreserpinized (controls)			Reserpinized			Increase of mean response during reserpinization	
		N of exp	Secretion in meq total acid		N of exp	Secretion in meq total acid		In meq total acid	In per cent of mean for controls
			Basal secretion	Response[1] to insulin hypoglycemia		Basal secretion	Respons to insulin hypoglycemia		
39	0 8	3	0 05	**2 44** 1 84–2 96	3	0 07	**1 83** [2]0 97–2 57	**-0 61**	-25
40	0 3	4	0 03	**3 46** 3.28–3 84	3	0 09	**2 45** 1 43–2.99	**-1 01**	-29
[3]41	0 5	5	0 04	**3 47** 1 59–4 83	3	0 12	**1.21** 0 83–1 56	**-2 26**	-65
45	0 3	7	0 05	**1 43** 0 84 3 33	3	0 37	**0 56** 0 21–0 80	**-0 87**	-61
48	0 5	4	0 03	**1.32** 0 61–1 98	3	0 23	**0 45** 0 39–0 51	**-0 87**	-66
78	0.5	3	0 00	**2 48** 2 14–0 06	3	0 08	**0 63** 0 3–0 72	**-1 85**	-75

[1] Correction made for basal secretion
[2] Range of secretory responses
[3] Illustrated in Fig 4

0 61 meq (cat 39) to 2 26 meq (cat 41) or from 25 (cat 39) to 75 per cent (cat 78) According to the analysis of variance the 3 hour secretory responses of nonreserpinized and reserpinized cats differed highly significantly ($P < 0\ 001$) Analysis of variance of the corrected 2 hour secretory responses gave results almost identical to those for the 3 hour responses The curve for acid output during the 3rd postinjection hour had thus approximately the same shape in nonreserpinized and reserpinized cats

In 3 animals (cats no 40 41 and 45) the mean blood sugar level both when starving and during maximum hypoglycemia was elevated during reserpinization by 15 to 30 mg glucose per 100 ml blood Elevation in the remaining cats (no 39 48 and 78) was less than 15 mg glucose per 100 ml blood The elevated blood sugar level during reserpinization is probably not entirely responsible for the lowered secretory responses of reserpinized cats to insulin hypoglycemia as emerges from the following observations

(1) In cats no 39 48 and 78 the mean blood sugar level was only slightly affected by reserpinization The reduction of the mean secretory responses to insulin hypoglycemia was however comparable to that of the remaining 3 cats

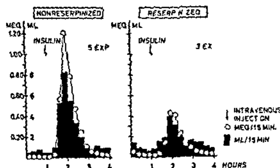


Fig 4 Basal gastric secretion and secretory respons (uncorrected) to insul n hypoglycemia in a gastric fistula cat (no 41) nonreserpinized (mean of 5 exp) and reserpinized (mean of 3 exp) Dose of insulin 0 5 I U per kg of body weight i v

(2) Comparing in the same animal experiments with approximately equal initial blood sugar levels and equal levels during maximum hypoglycemia (difference less than 9 mg glucose per 100 ml blood), there was still an obvious difference in the secretory responses of the nonreserpinized and reserpinized animal

(3) In experiments with approximately equal hypoglycemic reaction (difference in blood sugar fall less than 8 mg per 100 ml blood) the secretory response of the nonreserpinized animal always exceeded the response of the same but reserpinized animal

The vehicle for Serpedin® was injected intramuscularly daily for 15 days in two cats in a volume corresponding to that for reserpine in 4 experiments on each animal no change was observed either in basal secretion of acid or in the secretory responses to histamine

Two animals succumbed in connection with reserpinization one animal 36 hours the other 3 days after the last reserpine injection In the former animal an ulcer was found just proximal to the pyloric sphincter and in the same region a small muccosal erosion was observed in both animals Neither animal showed macroscopic ulcers in the duodenum

Discussion

It has previously been reported that in nonanesthetized gastric fistula cats hourly repeated infusions of histamine or gastrin produced fairly uniform acid secretory responses provided that the initial response (uncorrected) was less than 0 75 meq of total acid per hour (EMÅS 1960 b) These findings appear to apply also to reserpinized cats In the experiments with methacholine the increased salivation produced by this drug reduces the usefulness of the gastric fistula preparation but the results have been included for the reasons already mentioned As reported al o by FRIEDMAN (1950) insulin hypoglycemia elicited acid secretion in nonanesthetized cats

Reserpinization of the cats elevated basal secretion the secretory responses to histamine and gastrin were increased while that to methacholine remained unchanged and that to insulin hypoglycemia was reduced The magnitude of the increase or reduction was not related to the length of the reserpinization

period Both the increases and the reduction of gastric secretion were highly significant As already mentioned the reduction may be partly but probably not entirely ascribable to the elevated blood sugar levels of reserpinized cats A slight hyperglycemia has been reported also in rabbits and dogs following a single reserpine injection of 0 1 mg per kg of body weight (KUSCHKE and FRANTZ 1955) The essential point however is not that the secretory responses to insulin hypoglycemia are decreased but that reserpinization produces no increase Differences among or within animals in e g sensitivity to reserpine or to the secretory stimuli used cannot be responsible for the dissimilar effects of reserpinization on gastric secretion, because all the animals used in the experiments with methacholine and insulin revealed markedly increased responses to histamine and/or gastrin Furthermore when methacholine and insulin were tested either histamine or gastrin had been used within the same period of reserpinization What effect reserpinization exerts on the acid secretory response thus depends on the stimulus by which the secretion is elicited

The elevation of basal secretion produced by reserpinization is in agreement with the findings of LIEBOWITZ and CARBONE (1957) HAVERBACK and WIRTSCHAFTER (1962) noticed normal secretory response to histamine in a human being treated with reserpine for 10 days The dose of reserpine administered per kg of body weight was however considerably less (1 mg daily intramuscularly as the total dose) than in the experiments reported here

Reserpine depletes the stores of catecholamines from adrenergic nerve terminals (BERTLER CARLSSON and ROSENGREN 1956 MUSCHOLL and VOGT 1958) and from the brain (HOLZBAUER and VOGT 1956) To what extent the effects of reserpinization on gastric secretion may depend upon reduced or abolished inhibitory actions of the adrenergic nerves on the stomach is a matter now being studied

Reserpine releases serotonin (PLETSCHER SHORE and BRODIE 1955) which is normally present in a relatively high concentration in the gastric and duodenal mucosa (FELDBERG and TOH 1953) Parenterally administered serotonin is reported to inhibit the gastric acid secretion induced by *inter alia* histamine (BLACK FISHER and SMITH 1958 de CORRAL SALETA 1960) and urecholine (HAVERBACK BOGDANSKI and HOGBEN 1958) and the serotonin precursor 5-hydroxytryptophan the secretion induced by insulin hypoglycemia (HAVERBACK *et al* 1958) It has been suggested that serotonin might be concerned with the physiological regulation of gastric secretion (RESNICK and GRAY 1961) but there is no proof of this In unpublished experiments (EMAS and THAM), reserpine treatment of cats for 3 or 4 days see METHODS) did not alter the serotonin content of the mucosa from the body and the antral part of the stomach The serotonin content in the venous blood of the reserpinized cats was too small to be estimated quantitatively by the direct method of WEISSBACH WAALKES and UDENFRIEND 1958 but it appeared to be less than in untreated cats (1 0 to 1 5 μg per ml blood It is impossible yet to decide

whether or not the reduction or elimination of the inhibitor serotonin from the blood may be responsible for the hypersecretion of reserpinized cats.

Several symptoms of the reserpinized cat indicate a parasympathetic predominance, e. g. miosis, bradycardia, diarrhea. Vagal impulses act synergistically with gastrin on the parietal cells (UVNÄS 1942, OLBE 1963) and a similar synergism is assumed to exist between local cholinergic reflexes and gastrin as well as histamine (GROSSMAN 1961). In the experiments presented here the increased responses of the reserpinized cats to histamine and gastrin and the elevated basal secretion might be attributable to a parasympathetic predominance in the reserpinized animal which increases the excitability of the parietal cells to histamine and gastrin.

Cholinergic drugs (LANGLOIS and GROSSMAN 1950, BURSTALL and SCHOFIELD 1954) and vagal impulses (for references see SCHOFIELD 1960) are considered to elicit acid secretion partly through the release of gastrin. One would therefore expect the "gastrin component" of the secretory responses to methacholine and insulin hypoglycemia to increase in the reserpinized cats as do the responses to intravenous gastrin. The responses to methacholine were, however, essentially unaffected by reserpinization while those to insulin hypoglycemia were reduced. These results appear inconsistent with the explanation proposed for the increased responses of reserpinized cats to histamine and gastrin, unless methacholine and insulin hypoglycemia release lesser amounts of gastrin in reserpinized than in nonreserpinized cats. This latter problem is under investigation and experiments so far have demonstrated a pronounced reduction of the antral gastrin activity in cats treated with reserpine (EMÅS and FYRÖ to be published).

To conclude, a clear picture of the mode of action of reserpinization on gastric secretion cannot be obtained from the data presented here. The results indicate a complex action on the gastric secretory mechanism. Histamine and gastrin are considered to activate gastric secretion at the parietal cell level. The heightened secretory responses of reserpinized cats to histamine and gastrin are therefore suggestive of an increased excitability of the parietal cells to these stimuli, which may be caused either by a reduction or loss of inhibitory influences or by an increase of stimulating factors. Methacholine and insulin hypoglycemia are considered to elicit gastric secretion partly by the release of gastrin. The unaffected and reduced responses of reserpinized cats to methacholine and insulin hypoglycemia may suggest that reserpinization also interferes with the gastrin mechanism.

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Chemical and Histochemical Evaluation of the Distribution of Catecholamines in the Rabbit and Guinea Pig Hearts

By

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Abstract

ANGELAKOS, E T K FUXE and M L TORCHIANA *Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea pig hearts* Acta physiol scand 1963 *59* 184—192 — Chemical *determinations of noradrenaline (NA) adrenaline (A) and dopamine* (DA) were made in the right and left atria and ventricles (RA LA RV LV) of rabbit and guinea pig hearts The concentration of these amines was also studied in the sino-auricular (SA) node of the rabbit Some of these tissues were also examined for the distribution of catecholamines using a histochemical fluorescence technique NA was the *predominant catecholamine present in atria and ventricles It was* more concentrated in the RA than LA and in the RV than in the LV of the rabbit and guinea pig In the latter NA was distinctly higher in the atria than in the ventricles By contrast A was more concentrated in ventricles than in atria of both species and accounted for about 5—10 per cent of the total catecholamine content DA was found pre*dominantely in the atria more in RA than in LA High concentrations* of DA were found in the SA node region Histochemical studies on rabbit tissues showed that the catecholamines were found within nerve structures A high density of fluorescent fibers and fiber bundless was found in the SA node region An excellent correlation between the histochemical and chemical findings was obtained when both NA *and DA were considered*

Table I Noradrenaline (μ /g) in the right and left atria of the rabbit (10 animals)

	Right atrium	Left atrium
Mean	1.90	1.12
SD	± 0.65	± 0.20
SE	± 0.21	± 0.06

Noradrenaline (NA) has been found to occur in the heart of many species studied The literature has been reviewed by EULER (1956) and more recently by UDENFRIEND (1962) Few systematic studies have been made on the distribution of NA in various parts of the heart SHORE *et al* (1958) reported that in the dog heart the NA content was higher in the atrial than in ventricular tissue but they found no significant difference between the right atrium and the SA node Later SERRANO *et al* (1960) using somewhat different methods in the same species obtained similar results regarding the distribution of total catechol amines in the atrium and ventricle but a higher concentration in the SA node region than in the rest of the right atrium The latter was attributed to a higher concentration of adrenaline (A) in the SA node region

Recently a new histochemical technique has been developed for the identification of catecholamines based on the formation of highly fluorescent condensation products with formaldehyde (FALCK 1962 FALCK *et al* 1962) This method has been used for the cellular localization of monoamines in the adrenal medulla brain and adrenergic fibers in various organs and tissues (FALCK 1962 FALCK and TORP 1962 CARLSSON *et al* 1962) However no systematic studies have been made with the heart It was therefore of interest to evaluate the distribution of catecholamines in cardiac tissue using chemical determinations and to correlate some of the findings with the histochemical fluorescence technique

Methods

For all determinations the hearts were removed rapidly from the animal after sacrifice For the chemical assays the tissues were blotted dry weighed and placed in 10 per cent trichloracetic acid After mincing and filtering 0 3 ml of 10 per cent EDTA was added and the NA and A determinations were made according to the method of EULER and LISHAJKO (1961) Briefly this method involves adsorption on alumina at pH 8 5 elution with 0 25 N acetic acid and oxidation at pH 6 5 with potassium ferricyanide for 3 min followed by sodium hydroxide ascorbic acid ethylenediamine mixture The amounts of NA and A were calculated from fluorimetric readings at 395/490 and 435/540 mμ A Coleman fluorimeter or an Aminco spectrofluorimeter were used

In some cases separate samples were oxidized at pH 3 5 for 2 min the rest of the procedure remaining the same Under these conditions practically all of A but very little of NA present is oxidized (EULER and HAMBERG 1949) When these samples were

Table II Distribution of NA (μg/g) in various parts of the right atrium (2 atria pooled for each determination)

	Node	Right atrium		Total	Left atrium Total
		Body	Appendage		
	0.99	1.88	3.67	2.30	1.96
	0.97	0.83	2.48	1.36	1.03
	—	1.78	2.58	2.05	1.49
	—	1.35	1.90	1.67	1.36
Mean	0.96	1.47	2.66	1.85	1.1[illegible]

read at 435/540 mμ the ratio of fluorescence for A and NA was of the order of 300—500 1 Thus very small amounts of A could be determined reliably even in the presence of high concentrations of NA

DA was determined by the method of CARLSSON and WALDECK (1958) after adsorption on alumina Briefly the method involves oxidation with iodine acidification and exposure to UV light

In the histochemical studies tissues were prepared as described by FALCK (1962) This method is based on the development of fluorescence when certain monoamines condence with formaldehyde The specificity of the method has been studied by FALCK *et al* (1962)

The SA node region at the cavo appendicular angle was identified by gross observation by its distinct whitish appearance and its pacemaker function This was repeatedly confirmed in several histological sections stained with hematoxyline eosine

Results

A total of 10 pairs of left and right atria were analysed for NA and A The data for NA are given in Table I These tissues were found to be virtually free of A (less than 1 per cent of NA content) It is clear that in all cases the NA concentration was higher in the right than in the left atrium Further fractionation using tissues pooled from 2 animals for each determination indicated that the concentration of NA was higher in the right atrial appendage (auricle) than in the body of the right atrium (Table II) As is evident from the tabulated values the concentration of NA in and around the region of the SA node was not higher A separate set of 5 determinations gave a value of 0 7 μg/g Generally the amount found in the nodal area per gram of tissue was lower However this is probably due to the fact that a great deal of extraneous tissue such as part of the left superior vena cava and surrounding fat were included in these initial studies in order to insure that the entire nodal tissue was removed

To clarify the significance of these observations the histochemical fluorescence technique was used Tissues from the left and right atrial appendage from 9 rabbits were examined In all cases it was found that the tissues contained a

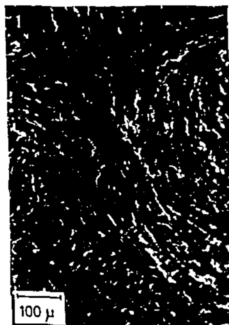






Fig 1 Right atrium of rabbit heart The fluorescent material is localized to varicose fibers of which most have been cut longitudinally (Magnification 165

Fig 2 Left atrium of rabbit heart The fluorescent material is localized to varicose fibers of which most have been cut longitudinally The density of the fibers is much lower than in the right atrium (Magnification 16)

Fig 3 Sino-auricular node of rabbit heart The fluorescent material is localized mainly to fiber bundles, forming a dense network infiltrating the nodal region (Magnification 165 ×)

Table III Catecholamines (μg/g) in various parts of the rabbit heart (pooled tissues)

Tissue	Group	Number of prep	wt (g)	NA	A	DA
Node	I	5	0 106	2 04	< 0 01	2 07
	II	5	0 048	2 81	< 0 01	6 00
	III	8	0 132	3 03	< 0 01	2 18
Right atrium	I	5	0 487	2 81	0 07	0.23
	II	5	0 303	4 52	< 0 01	0 [illegible]5
	III	8	0 821	3 11	< 0 01	0 40
Left atrium	I	5	1 01	1 53	< 0 01	< 0 01
	II	5	0 56	1 86	< 0 01	0 07
Total atria	I	5	1 61	1 95	0 0[illegible]	0 [illegible]1
	II	5	0 94	2 71	< 0 01	0 43
Right ventricle	I	5	3 06	3 24	0 12	0 10
	II	5	2.25	3 03	0 14	0 05
Left ventricle	I	5	12 90	1 75	0 04	0 03
	II	5	11 66	1 95	0 09	0 02
Total ventricles	I	5	15 96	2 04	0 06	0 05
	II	5	13 88	[illegible] 12	0 11	0 03
Total heart	I	5	17 57	2 03	0 06	0 0[illegible]
	II	5	14 84	2 16	0 11	0 0[illegible]

Right atrium less the SA node
Calculated value including both atria and the SA node
Calculated value
Value checked with oxidation at pH 3 5 for 2 min and reading at 435/540 mμ

network of nerve fibers exhibiting strong fluorescence These fibers and fiber bundles were distributed throughout the tissue but in all specimens examined the density of the fluorescent fibers was much greater in the right than in the left atrial appendage and greatest near the SA node (Fig 1—3)

The SA node region contained fluorescent fibers and especially fiber bundles of a density that has not been observed in any other tissue These fibers and fiber bundles formed a very dense meshwork *infiltrating the node region* (Fig 3) *Occasionally this meshwork was separated into several small* distinct patches

Careful examination of many sections through the SA node region and atrial tissue did not reveal the presence of any fluorescent cells suggestive of catechol containing chromaffin cells as those found in the adrenal medulla and sympathetic ganglia (FALCK 1962 FALCK and TORP 1962)

In the left ventricle the density of the fluorescent fibers was about the same as in the left atrium There was no evidence of any higher density of fluorescent

Table IV Catecholamines (μg/g) in various parts of the guinea pig heart (pooled tissues from 5 animals)

Tissue	wt (g)	NA	A	DA
Right atrium	0.166	4.63	0.99	1.39
Left atrium	0.228	3.11	< 0.01	0.83
Total atria	0.394	3.75	0.04	1.07
Right ventricle	1.28	2.21	0.31	0.08
Left ventricle	6.13	1.96	0.25	0.06
Total ventricles	7.41	2.01	0.76	0.05
Total heart	7.80	2.10	0.25	0.11

Right atrium including the SA node.
Calculated value including both atria and the SA nod
Calculated value
Value checked with oxidation at pH 3.5 for 2 min and reading at 435/540 mμ

fibers near or around the purkinje tissue in association with the papillary muscles and endocardial layers The location of purkinje tissue was identified with the periodic acid Schiff technique

Two animals were treated with reserpine (1 mg/kg for 2 days) and sections from the right and left atrium and the SA node were examined histochemically No fluorescent material could be found in any of these sections The same tissues were analyzed chemically and no catecholamines were detected within the limits of the sensitivity of the method (less than 0.01 μg/g)

In order to extend these observations and to resolve the discrepancy between the chemical and histochemical findings regarding the nodal region tissues from several rabbits were pooled and the catecholamine content was determined as previously In this case analyses were also made on the right and left ventricle Furthermore the content of dopamine (DA) was also determined using the method of CARLSSON and WALDECK (1958) Pooling the tissues from several animals was essential in order to obtain an accurate estimate of the content of A and DA In this group of experiments the SA node was dissected carefully so that little or no extraneous tissue was included (weight 10—20 mg/node) The results from 3 experiments using tissues from 5 to 8 animals each are shown in Table III They show that DA or a DA like compound is concentrated in the SA node region A brief report of this observation has been presented (ANGELAKOS and TORCHIANA 1963)

Finally a similar experiment was made using tissues pooled from 5 guinea pigs The results are shown in Table IV In this case it was not possible to identify with certainty and separate the SA node and it was therefore included in the right atrium

Discussion

It is clear from the results that in the rabbit heart the right atrium(RA) contains more NA than the left atrium (LA) However, the SA node region contains the same amount or less NA than the remaining part of the RA This is in agreement with the findings of SHORE et al (1958) in the dog heart However SHORE et al (1958) found no differences in the NA content between the RA and LA in the dog and this agrees with the results of CHIDSEY et al (1962) obtained in the same species

The NA concentration is highest in the right ventricle (RV) The concentration in the entire heart found in the present studies (about 2 μg/g) is similar to that reported by SHORE and OLIN (1958) but higher than that given by HÖKFELT (1951) for the heart of the same species

A is very low or absent from the rabbit atria but present in small but definite concentrations in the ventricles Again the RV contains more on a μg/g basis than the LV By contrast DA is found in larger concentrations in the atria than in the ventricles It is evident that the RA contains more DA than the LA and that this amine is clearly concentrated in the SA node region (ANGELAKOS and TORCHIANA 1963)

It is difficult to correlate the present observations with the findings of SERRANO et al (1960) in the dog especially since their results differ in many significant aspects from those reported by SHORE et al (1958) in the same species Since SERRANO et al (1960) used iodine for oxidation it is possible that the higher concentration of total catecholamines in the SA node region reported by these authors reflects the combined amount of NA and DA present However these authors concluded that the concentration of A was higher in the SA node region than in the rest of the atrium and higher in the atria than in the ventricles These observations in the distribution of A are not in agreement with those of SHORE et al (1958) in the same species and contrast with the present observations in rabbits and guinea pigs

Fluorescence histochemistry indicates that the catecholamine content of the atria is confined within nerve structures The density of these fibers is greater in the RA than in LA in agreement with the chemical findings on the concentration of NA and DA Comparison of the results of the two methods indicates that the high density of the fluorescent fibers observed in the SA node region must contain predominantely DA Thus when all the catecholamines are considered the results indicate an excellent correlation between the histochemical and chemical methods

The supply of autonomic nervous system fibers in the various parts of the heart has been studied repeatedly with conventional histological techniques (BLAIR and DAVIES 1934–35 NONIDEZ 1943 TCHENG 1951) and more recently with the electron microscope (TORII 1962) For the most part previous studies

could not identify with certainty the adrenergic fibers as it is possible with the fluorescence technique

The findings in the guinea pig are similar to those in the rabbit in most of the essential details Although the data collected in this species are limited it is clear that the RA and RV contain higher concentrations of NA than the corresponding left tissues In this species the concentration of NA in the atria is clearly higher than in the ventricles This is similar to findings in the dog (SHORE et al 1958) The values given here for the right atrium and total heart agree closely with those reported by CROUT et al (1962) and UDENFRIEND (1962) but are higher than the values given by PEPEU et al (1961) for this species The concentration of A is again higher in the ventricles than in the atria and it is about twice as much in the guinea pig as in the rabbit The high concentration of DA in the RA which included the SA node suggests that in this species also DA is concentrated in the nodal region However direct proof of this is lacking

The authors wish to express their appreciation to Professors U S von Euler and N Å Hillarp for their advice given during the course of these investigations This work was supported by grants from the Tobacco Industry Research Council U.S Public Health Service Career Development Award (GM K3 15457) Training Grant (HTS 5836) and U S Public Health Service Grant (NB 07854 03)

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Addendum

Since the preparation of this manuscript two recent reports have come to our attention Muscholl (*Naunyn-Schmied be g s* Arch. exp Path Pharmak. 1959 *237* 350) studied the distribution of NA and A in the heart of cats rabbits and rats. His results agree closely with those reported here Mastsuo (Jap J Pharmacol 1962 *12* 62) also noted a d fference in the NA content between the right and left atrium of the rabbit

Acta physiol scand 1963 59 193—198

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Effect of Glucose and Sodium Succinate on Oxygen Uptake and Histamine Release in Rat and Guinea-Pig Lung Tissue in Vitro

By

BERTIL DIAMANT and BO FREDHOLM

Received 18 January 1963

Abstract

DIAMANT B and B FREDHOLM *Effect of glucose and sodium succinate on oxygen uptake and histamine release in rat and guinea pig lung tissue in vitro* Acta physiol scand 1963 *59* 193—198 — Previous reports on the effect of sodium succinate and glucose on histamine release and oxygen uptake in guinea pig and rat lung tissue have been somewhat conflicting and difficult to interpret The present study was undertaken to determine whether the effect of the aforementioned substrates on histamine release could be correlated to their effect on the oxygen uptake The results have shown that such a correlation exists in some but not all of the reactions investigated The hazards of interpreting correlations between oxygen uptake and histamine release in lung tissue are pointed out.

The enhancing effect of sodium succinate on anaphylactic histamine release from minced guinea pig lung tissue has repeatedly been correlated to its stimulating effect on oxygen uptake (MOUSSATCHÉ and PROUVOST DANON 1958 YAMASAKI MURAOKA and ENDO 1960 CHAKRAVARTY 1962) This correlation has in fact been one of the circumstantial proofs given of anaphylactic histamine release from such tissue being an energy requiring reaction

The effect of glucose on oxygen uptake and on anaphylactic histamine release from guinea pig lung tissue varies in different investigations Thus YAMASAKI MURAOKA and ENDO (1960) noted a slight increase in histamine release as well as in oxygen uptake CHAKRAVARTY (1962) reported no effect on histamine release although the oxygen uptake was statistically increased in 2 out of 3 ex

Table I Histamine release induced by antigen from guinea pig lung tissue and by antigen and compound 48/80 from rat lung tissue under oxygen with and without glucose (5 6 mM) in the incubation medium Values denote histamine release in % of total histamine content

	Histamine release under oxygen		
	No substrate (A)	Glucose (B)	Difference B—A in individual experiments (Mean ± SE)
Guinea pig			
Antigen	21 7 (7)	31 5 (7)	¹10 3 ± 0 9
Rat			
Antigen	23 9 (8)	30 9 (8)	¹7 0 ± 1 2
Compound 48/80	26 6 (8)	32 3 (8)	¹6 ± 0 9

Bracketed figures denote the number of experiments

¹ Differs from zero P < 0 001

periments and DIAMANT (1962 a) observed an increase (statistically significant) in histamine release without measuring the oxygen uptake Histamine release from rat lung tissue induced by compound 48/80 as well as by antigen was also found to be stimulated by glucose in the presence of oxygen (DIAMANT and UVNÄS 1961 DIAMANT 1962 b)

Whereas glucose has been observed to enhance histamine release in the presence of oxygen from both guinea pig and rat lung tissue, sodium succinate is effective only in the guinea pig (DIAMANT 1962 b)

The aim of the present investigation was to evaluate whether or not the effect of glucose and sodium succinate on histamine release could be correlated to the metabolic reactions occurring in lung tissue as judged by the oxygen uptake

Methods

Histamine release

Rat and guinea pig lung tissue was prepared and incubated under oxygen as previously described (DIAMANT and UVNÄS 1961) Histamine release was induced by antigen (crystallized egg albumin) 1 mg/ml or compound 48/80 35 µg/ml The concentration of both glucose and sodium succinate was 5 6 mM Histamine release was computed as a percentage of the total histamine content with deduction of the spontaneous histamine release As demonstrated earlier (DIAMANT 1962 a th spontaneous histamine release is the same under the various experimental conditions

Manometric procedure

The oxygen uptake was measured in a Warburg apparatus at 37 C The preparation of the lung tissue was identical with that in the experiments on histamine release and the same phosphate medium (pH 7 1—7 2) was used for incubation Each flask con

Table II Increase produced by glucose (5 6 mM) sodium succinate (5 6 mM) and glucose plus sodium succinate in anaphylactic histamine release from guinea pig lung tissue under oxygen Values denote differences from histamine release without substrate in % of total histamine content

	Histamine release under oxygen		
	Glucose (A)	Succinate (B)	Glucose + Succinate (C)
Guinea pig			
Antigen	8 8	17 5	21 8
(4 expts)	9 3	18 0	24 4
	9 5	14 5	20 7
	10 9	12 9	23 5
Mean ± SE	9 6 ± 0 5	15 7 ± 1 ?	2? 6 ± 0 8

Significance of difference of means B—A 0 001 < P < 0 01 C—B 0 001 < P < 0 01

tained 3 0 ml of phosphate medium 100 mg (wet weight) of lung tissue and — when tested — glucose and/or sodium succinate (5 6 mM of each final concentration) 0 2 ml of 10 % KOH was present in the centre well The flasks were filled with 100 % oxygen The rate of oxygen uptake was constant over a period of at least 2 hours and readings were made at 15 min intervals Q_O was calculated from the one hour value and the dry weight of the tissue sample

Results

Histamine release

The effect of glucose on histamine release under oxygen induced by antigen from rat and guinea pig lung tissue as well as by compound 48/80 from rat lung tissue was re calculated from new and earlier published experiments (Table I) The increase due to glucose was highly significant in all reactions

Antigen induced histamine release from guinea pig lung tissue was increased more by sodium succinate than by glucose The increase produced by sodium succinate plus glucose was greater than that produced by sodium succinate alone (Table II)

Histamine release from rat lung tissue induced by antigen or compound 48/80 was not affected by sodium succinate (Table III)

Oxygen uptake

In both species the oxygen uptake was increased in the presence of glucose Sodium succinate enhanced the oxygen uptake still more When both substrates were present there was no further increase in oxygen uptake as compared to that in the presence of succinate alone (Table IV)

Table 1 *Histamine release induced by antigen from guinea pig lung tissue and by antigen and compound 48/80 from rat lung tissue under oxygen with and without glucose (5 6 mM) in the incubation medium Values denote histamine release in % of total histamine content*

	Histamine release under oxygen		
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Rat			
Antigen	23 9 (8)	30 9 (8)	[1]7 0 ± 1 2
Compound 48/80	26 6 (8)	32 3 (8)	5 6 ± 0 9

Bracketed figures denote the number of experiments

[1] Differs from zero P < 0 001

periments and DIAMANT (1962 a) observed an increase (statistically significant) in histamine release without measuring the oxygen uptake Histamine release from rat lung tissue induced by compound 48/80 as well as by antigen was also found to be stimulated by glucose in the presence of oxygen (DIAMANT and UVNAS 1961, DIAMANT 1962 b)

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The aim of the present investigation was to evaluate whether or not the effect of glucose and sodium succinate on histamine release could be correlated to the metabolic reactions occurring in lung tissue as judged by the oxygen uptake

Methods

Histamine release

Rat and guinea pig lung tissue was prepared and incubated under oxygen as previously described (DIAMANT and UVNAS 1961) Histamine release was induced by antigen (crystallized egg albumin) 1 mg/ml or compound 48/80 35 μg/ml The concentration of both glucose and sodium succinate was 5 6 mM Histamine release was computed as a percentage of the total histamine content with deduction of the spontaneous histamine release As demonstrated earlier (DIAMANT 1962 a the spontaneous histamine release is the same under the various experimental conditions

Manometric procedure

The oxygen uptake was measured in a Warburg apparatus at 37 C The preparation of the lung tissue was identical with that in the experiments on histamine release and the same phosphate medium (pH 7 1—7 2) was used for incubation Each flask con

Discussion

The results have shown that a correlation exists between the enhancing effect of glucose on the oxygen uptake and on the histamine release in guinea pig and rat lung tissue. This correlation is also valid for the effects of sodium succinate in the guinea pig. So far the results are in agreement with the view that histamine release depends on the metabolic reactions in the tissue as judged by the oxygen uptake.

In guinea pig lung tissue glucose together with sodium succinate enhanced antigen induced histamine release more than did sodium succinate alone. This increase in contrast to the aforementioned was not reflected in the rate of oxygen uptake.

In rat lung tissue sodium succinate had no effect on the histamine release induced by antigen or by compound 48/80 although the oxygen uptake was enhanced to the same extent as in guinea pig lung tissue.

Consequently it seems that an increased oxygen uptake does not necessarily represent an increased provision of cellular energy available to the histamine releasing reactions.

These discrepancies together with the observations that other metabolites of the Krebs cycle did not enhance the anaphylactic histamine release from guinea pig lung tissue although the oxygen uptake was highly increased (YAMASAKI *et al.* 1960, AUSTEN and BROCKLEHURST 1961, CHAKRAVARTY 1962) cast some doubt on the relevancy of correlating the oxygen uptake to the histamine release. Furthermore it is generally agreed that histamine release occurs from the mast cells. These cells constitute an exceedingly small part of the lung tissue and it is improbable that metabolic reactions taking place in these cells would be reflected in the oxygen uptake of the tissue as a whole. This also implies that the observed correlations between histamine release and oxygen uptake of whole tissue might be coincidences.

One way of attacking this problem would be to repeat these experiments with a pure mast cell population. In our view however a correlation under these premises should be considered only as circumstantial evidence of histamine release being an energy requiring process. Although much evidence of this kind has been put forward in favour of this hypothesis the final proof will depend on correlations between histamine release from mast cells and their content and turnover of energy rich phosphate bonds. This seems especially important in view of recent investigations indicating a significant role of anaerobic glycolysis for histamine release and mast cell degranulation (for references see DIAMANT 1962 b).

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Acta physiol scand 1963 59 199—220

From the Institute of Neurophysiology University of Copenhagen Denmark

Refractory Period and Conduction Velocity of the Striated Muscle Fibre*

By

FRITZ BUCHTHAL and LISE ENGBÆK

Received 11 February 1963

Abstract

BUCHTHAL F and L ENGBÆK *Refractory period and conduction velocity of the striated muscle fibre* Acta physiol scand 1963 59 199—220 — Refractory period and conduction velocity of transmembrane potentials were determined in single frog muscle fibres by intracellular stimulation and recording at 14 20 and 25 C At the end of the absolutely refractory period the latency of the potential evoked by the second stimulus was substantially increased mainly due to a reduction in conduction velocity delayed firing causing at most 10 per cent of the total delay The absolutely refractory period terminated at the onset of the negative afterpotential recovery in excitability amplitude and conduction velocity in the relatively refractory period occurred within a time interval in which the membrane potential did not change more than about 3 mV At 2—3 times the absolutely refractory period excitability and conduction velocity had a supernormal phase At the absolutely refractory period the level of depolarization required to initiate a propagated response had increased from 40 to more than 60 mV (recorded 0 25—0 6 mm from the stimulating electrode) A local response appeared at a time interval of 70—80 per cent of the absolutely refractory period of the propagated response

Most of the information on the refractory state derives from studies of heart muscle (for references see SCHUTZ 1958 and HOFFMAN and CRANEFIELD 1960) and of myelinated nerve The amplitude of the nerve action potential is reduced considerably at the end of the absolutely refractory period (TASAKI and TAKEUCHI 1942) The test potential is delayed due to a reduction in the conduction

* Zum 60 Geburtstag von Herrn Professor A von Muralt

velocity (GASSER and ERLANGER 1925) or to prolonged latency as well (GOTCH 1910 FORBES, RAY and GRIFFITH 1923) In the later part of recovery there is at any rate in nerve with a curtailed negative afterpotential a transient increase in excitability (GASSER and ERLANGER 1930)

In skeletal muscle the amplitude of the potential evoked by the test stimulus was reduced to less than 25 per cent at the end of the absolutely refractory period (GLASER and STARK 1958) This could be due to the activation of fewer fibres since the amplitude of the potential recorded from directly stimulated small fibre bundles in human muscle was 90 per cent of the conditioning potential (FARMER BUCHTHAL and ROSENFALCK 1960)

Therefore to determine the absolutely refractory period it is necessary to record from the single muscle fibre To determine whether and to what degree the amplitude of the action potential is reduced it is necessary to record intracellularly the amplitude of the external potential reflecting more the rise time than the amplitude of the potential change At the same time this affords the possibility to relate the changes in excitability to the changes in membrane potential and to determine whether there is a supernormal phase in the recovery of skeletal muscle

LUCAS (1909) showed that the arrival of the potential evoked by the test stimulus was delayed at the end of the absolutely refractory period with indirect stimulation of single fibres KUFFLER (1942) found a decreased conduction velocity KOSTYAK 1958 recorded a delayed arrival of the intracellularly recorded action potential in the relatively refractory period

To determine whether the delay of the potential evoked by the test stimulus was due to diminished conduction velocity or to prolonged latency or to both we have recorded the action potential with two electrodes one so close to the stimulating electrode as to avoid conduction and one at a distance which allowed measurement of the time of conduction Thereby it was also possible to study a nonpropagated response in the absolutely refractory period

Methods

Experimental chamber and temperature regulation

The frog s sartorius muscle (*Rana temporaria* or *Rana esculenta*) was placed over the slightly convex bottom (0 5 mm thick) of a perspex chamber and was fixed at both tendon ends The degree of stretch usually about 30 per cent above equilibrium length was adjusted until stimulation of the single fibre no longer caused movements visible under the microscope The chamber was filled with Ringer s solution to about 0 5 mm above the surface of the muscle The composition of the Ringer s solution was 115 mM NaCl 2 mM KCl 1 8 mM $CaCl_2$ 2 0 mM Na_2HPO_4 and 0 5 mM NaH_2PO_4 to obtain a pH of 7 2—7 3

Constant temperature (± 0 1 C) was obtained by circulating water maintained at constant temperature in a reservoir through the double walls of the experimental chamber The temperature was measured by a thermistor (Standard Electric Type U) placed in the Ringer s solution close to the recording electrodes

Electrodes

Glass capillary electrodes filled with 3 M KCl were used for intracellular recording and stimulation The impedance of the recording electrode was 10—20 MΩ and of the stimulating electrodes 5—10 MΩ By passing current pulses of 10—20 μA and of 0 5—1 msec duration at a frequency of 5 per sec through the stimulating electrode as anode in Ringer's solution the impedances of different electrodes could be adjusted to about the same value This procedure rendered the impedance more stable during an experiment

Stimulation

Pairs of rectangular stimuli (pulse duration 200 μsec) separated by adjustable time intervals were delivered from two stimulators (American Electronic Model 104 A) The intensity of each stimulus of the pair could be varied independently The frequency at which paired stimuli were delivered was less than 1 per 5 sec

The outputs from the two stimulators to the intracellular electrode were added by the use of two output transformers whose secondaries were connected in series The indifferent silver-silver chloride electrode was placed in the experimental chamber 10 mm from the tendon end of the muscle and 25—50 mm from the points of recording The stimulating electrode was shielded by a thin metal tube which could be advanced to cover all except the distal three millimeters of the capillary electrode The screen and the use of difference amplification reduced the transmission of the stimulus to the recording electrode sufficiently to allow recording of action potentials at a distance of 0 2 mm from the stimulating electrode (FATT and KATZ 1951) The microelectrode used for stimulation was connected to the input of a cathode follower to measure the potential across the membrane while the electrode was inserted into the fibre and to ascertain that it was placed intracellularly

Measurement of the stimulating current

The impedance of the electrodes varied in an unpredictable way with the stimulating voltag Therefore the stimulus current was used as a measure of the strength of stimulation The current was measured by connecting the indifferent electrode to earth across a resistor of 3 000 ohms Stimulating current and voltage were about proportional up to an intensity twice the threshold current (0 5—2 μA) As the current was increased further the impedance of the electrode decreased

A preceding stimulus of high intensity might or might not increase the impedance for a subsequent stimulus by no more than 10 per cent for 1—20 msec

Recording

The membrane potential and the action potential were amplified by a d c difference amplifier with a three stage input cathode follower (CF) The input CF was pentode coupled the second CF of large transconductance drove the shield of the input cable and the third CF was output CF The equivalent input capacitance including cable electrode holder and electrode was 3—4 μμF the frequency response was slightly overdamped (damping factor 2) and 3 db down at 6 kcps with a 10 MΩ electrode (GULD 1962)

When recording simultaneously from two points of the same muscle fibre each recording electrode was connected by a separate cathode follower to one channel of the double beam cathode ray oscilloscope To measure the excitability and to determine the refractory period at different intensities of the stimulus the stimulating current was recorded on one beam simultaneously with the action potential on the other beam

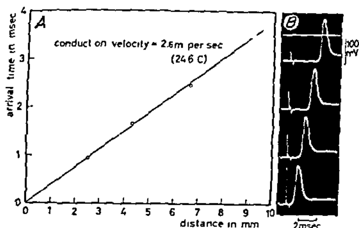


Fig 1 A Relation between the arrival time of the action potential (shown in B) and the distance from the point of stimulation at which it was recorded

Measurement of conduction velocity

The arrival time of the action potentials was used to determine the conduction velocity It was measured as the time interval between the onset of the stimulus and the intersection of the extrapolated steep part of the rising phase of the action potential with the base line Two series of experiments were performed

(1) The same recording electrode was inserted successively at 3—4 different distances from the stimulating electrode (Fig 1) The arrival time of the action potentials increased linearly between distances of 1 3 to 13 mm from the stimulating electrode *This indicates that the action potentials recorded at different distances originated from* the same fibre and that the conduction velocity is identical in different parts of the fibre

(2) The action potential was led off from the same fibre with two electrodes at different distances from the stimulating electrode This procedure was used also to determine the velocity of the potentials evoked by the test stimuli Assuming that the conduction velocity between the stimulating electrode and the recording electrode is the same as the velocity between the recording electrodes extrapolation to the point with zero arrival time indicates the border of the region of the fibre in which the action potential is initiated With intracellular stimulation at an intensity of 1 5 to 2 0 times threshold the extrapolated point of impulse initiation was displaced 0 3 ± 0 05 mm (13 fibres) from the stimulating anode towards the recording electrode Therefore the conduction velocity was measured only in those experiments in which the recording electrode was *more than 0 3 mm from the stimulating electrode* With a stimulus intensity between two and four times threshold the impulse was initiated as far as 0 6 mm from the stimulating electrode and only such experiments were included in which the closest recording electrode was more than 0 6 mm from the stimulating electrode

Extrapolation to the site where the action potential is initiated presupposes the same shape of the rising phase of the potential initiated by the stimulus without conduction as of the propagated potential This was the case as long as the stimulus intensity was 1.5 times threshold or more With stimulus intensities between 1 5 and 1 0 times threshold the latency of the potential evoked by the stimulus increased from 0 1—0 5 msec (20)

Placement of the electrodes

After insertion of a recording electrode the stimulating electrode was placed intracellularly at a suitable small distance Unless special precautions were taken the membrane potential measured by the recording electrode decreased by 1—5 mV when the stimulating electrode was inserted the greatest reduction occurring when the distance between the two electrodes was small This reduction was due to a short circuit of the membrane potential through the stimulating electrode and stimulator to ground When a condensor of 15 000 pF was inserted between stimulator and stimulating electrode the voltage drop was 0 5 to 1 mV immediately after insertion and was presumably due to leakage around the stimulating electrode This small change in voltage was convenient to indicate that stimulating and recording electrodes were placed in the same fibre and no further effort was made to abolish it

The distal recording electrode frequently had to be inserted into several fibres until an action potential was recorded by both electrodes When the electrode was situated in a fibre adjacent to the stimulated one a positive negative deflection not exceeding 2 mV was recorded This represents the extracellularly recorded potential of the stimulated fibre (EASTON 1955 KATZ 1956) Stimulation through an intracellular electrode as anode makes stimulation of adjacent fibres by external current spread unlikely The direction of the current is such that the adjacent parts of the membranes of neighbouring fibres are hyperpolarized while the strength of current has decreased appreciably where it has a depolarizing direction Moreover the identical threshold of the two simultaneously recorded action potentials indicated that the electrodes were in the same fibre and that only one fibre was activated

The distance between the stimulating and the recording electrodes was determined by means of a measuring microscope with an accuracy of 0 05 mm

Results

1 *The absolutely refractory period*

a) *Minimum time intervals*

The absolutely refractory period is the shortest interval between a conditioning stimulus and that supramaximal test stimulus which can evoke a propagated action potential It was obtained when the test current was 1 5 times the threshold current (Fig 2) produced by 1 4 to 1 5 times the threshold voltage At 1 2—1 3 times threshold the minimum time interval between effective stimuli was 20—10 per cent longer than the absolutely refractory period

The absolutely refractory period averaged 4 3 ± 0 06 msec at 14 C (39 fibres) 2 5 ± 0 05 msec at 21 C (58 fibres) and 2 1 ± 0 04 msec at 25 C (11 fibres) In fibres from another batch of frogs the refractory period was slightly longer 4 8 ± 0 11 msec at 14 C (11 fibres) 3 1 ± 0 10 msec at 20 C (15 fibres) and 2 2 ± 0 05 msec at 25 C (30 fibres Fig 3 B)

The absolutely refractory period of different fibres at four temperatures is presented in the histograms in Fig 3A There was no tendency for the fibres to fall in two groups one with short and one with long refractory periods as did small bundles of fibres in human muscle (FARMER *et al* 1960)

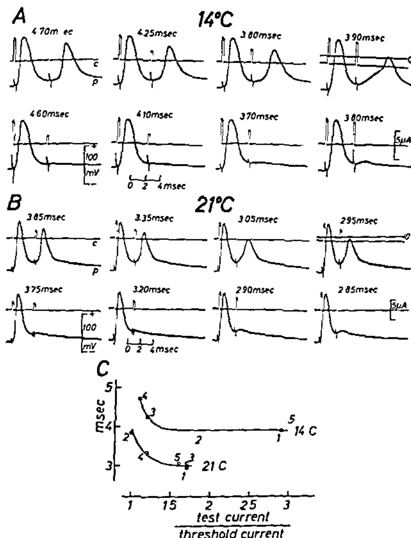


Fig 2 The refractory period at different intensities of the test stimulus

The upper records in A and B give the minimum time interval at which there was a propagated action potential (p) the lower records give the maximum time interval with just no response c is the stimulating current The distance between the stimulating and the recording electrodes was 1 5 mm

C. Refractory period as a function of the stimulating current in units of threshold current The figures at the points indicate the sequence of the recordings

b) *Rate of rise rate of fall and amplitude of the potential evoked by the test stimulus*

The rate of rise of the conditioning potential measured as the average slope between about 1/4 and 3/4 maximum amplitude was 500 V/sec at 25 C and 200—250 V/sec at 14 C The rising phase of the potential evoked by the test

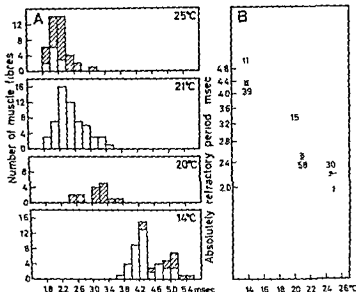


Fig 3 Absolutely refractory period at different temperatures

A In different fibres

B Average values with standard error of the mean the figures at the points indicate the number of muscle fibres

White columns (open circles) from first batch of frogs shaded columns (full circles) from second batch of frogs

stimulus at the absolutely refractory period was less steep (Fig 4A), slowest close to the point of stimulation Here it could be determined in eleven fibres the rate of rise was about 40 per cent of that of the conditioning potential measured 0 4—0 6 mm from the stimulating electrode In many experiments the rising phase was distorted by the stimulus The increase in the rate of rise of the potential evoked by the test stimulus depended critically on the potential s proximity to the absolutely refractory period In the example shown in Fig 4B the potential evoked precisely at the end of the refractory period reached its peak 0 9 msec after the test stimulus the potential evoked 0 1 msec later reached its peak 0 6 msec after the stimulus (25 C)

With increasing distance from the point of stimulation the rate of rise of the second potential approached that of the conditioning potential the second potential was conducted more slowly than the conditioning potential (p 10) giving more time for recovery in distal portions of the fibre Recorded 4—6 mm from the stimulating electrode the slope of the potential evoked by the test stimulus at the end of the absolutely refractory period was 300—400 V/sec at 25 C 150—200 V/sec at 14 C *i e* about 70 per cent of the slope of the conditioning potential

The maximum rate of fall was slower in the potential evoked by the test stimulus than in the conditioning potential the time to half peak being 50 to 100 per cent longer at 14 and 25 C Occasionally there occurred a hump of 1—2 mV 1 5—2 msec after the peak of the test potential (Fig 4Ab)

The amplitude of the conditioning potential was 110—130 mV the amplitude of the potential evoked by the test stimulus recorded 1 mm or less from

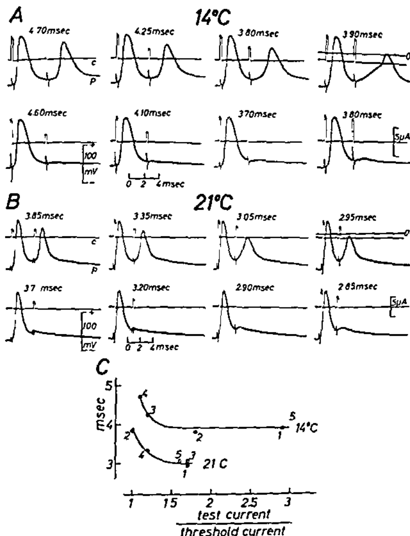


Fig 2 The refractory period at different intensities of the test stimulus

The upper records in A and B give the minimum time interval at which there was a propagated action potential (p) the lower records give the maximum time interval with just no response c is the stimulating current The distance between the stimulating and the recording electrodes was 1 5 mm

C Refractory period as a function of the stimulating current in units of threshold current. The figures at the points indicate the sequence of the recordings

b) *Rate of rise rate of fall and amplitude of the potential evoked by the test stimulus*

The rate of rise of the conditioning potential measured as the average slope between about 1/4 and 3/4 maximum amplitude, was 500 V/sec at 2ↄ C and 200—2ↄ0 V/sec at 14 C The rising phase of the potential evoked by the test

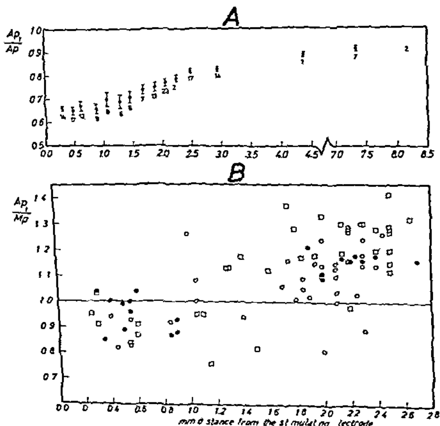


Fig 5 A The increase of the potential evoked by the test stimulus (Ap_1) at the end of the absolutely refractory period with increasing distance from the point of stimulation (14 20 and 25 C) Ap is the amplitude of the conditioning potential

The vertical bars indicate the standard error of the mean and the figures below the points the number of muscle fibres examined.

B Amplitude of the potential evoked by the test stimulus (Ap_1) at the end of the absolutely refractory period in units of the resting potential (Mp) with increasing distance from the point of stimulation (○ 14 □ 20 ● 25 C)

To ascertain that the potentials were propagated potentials recorded at less than 1.2 mm from the point of stimulation were simultaneously recorded at greater distances.

m/sec at 14 C (12 fibres) 2 3 ± 0 06 m sec at 20 C (21 fibres) and 2 8 ± 0 05 m sec at 25 C (39 fibres) (Fig 6B) The distribution of conduction velocities in different fibres at three temperatures is shown in the histogram of Fig 6A the variation (20—40 per cent) must be due in part to differences in fibre diameter since conduction velocity varied linearly with fibre diameter in isolated fibres HÅKANSSON 1956)

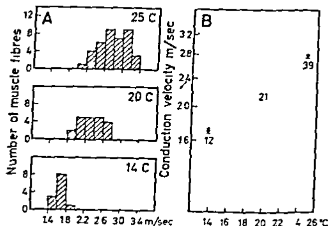


Fig 6 Conduction velocity at different temperatures

A In different fibres

B Average values with standard error of the mean the figures below the points denote the number of muscle fibres

In the same fibre subjected to gradual changes in temperature the conduction velocity between 1 7 and 2 7 m/sec varied proportionally with the rate of rise of the action potentials between 200 and 350 V/sec and linearly with the rate of fall and the duration of the action potential (6 fibres) With rates of rise of 400—500 V/sec the velocity was 2 7 and 2 8 m/sec (2 fibres) but the measuring accuracy did not allow to ascertain deviation from proportionality

Conduction was slowed substantially in the relatively refractory period At the end of the absolutely refractory period and at a distance of 4 5—6 mm from the stimulating electrode the latency of the potential evoked by the test stimulus was increased by about 50 per cent i e it was 1 1 ± 0 1 msec longer than the latency of the conditioning potential (25 C 12 fibres measured from peak to peak Fig 4A 4B and Fig 10A) At a distance of 0 6 mm or less from the stimulating electrode the onset of the potential could not be determined with accuracy The onset is probably slightly delayed as compared to that of the conditioning potential since the rate of rise is decreased and the latency was 0 1 ± 0 04 msec longer than the latency of the conditioning potential (25 C, 12 fibres measured to peak) Thus even though there may be a slight delay at the onset of the potential evoked by the test stimulus (less than 10 per cent of the total delay) the main cause of the delayed arrival at the more distant electrode is a decrease in conduction velocity

A plot of arrival times of the action potentials recorded at different distances from the point of stimulation shows that this reduction is most pronounced at 1—2 mm from the point of stimulation and approaches the velocity of the conditioning potential at about 5 mm from the point of stimulation (Fig 7) The slowing closer to the point of stimulation allows recovery to proceed ahead of propagation The average reduction in velocity to a distance of 5 mm from the point of stimulation was 35—45 per cent (Table I)

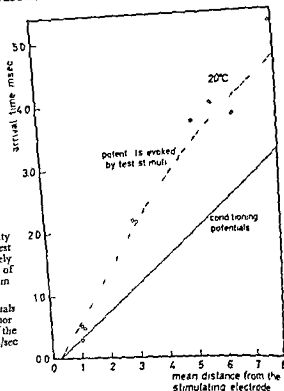


Fig 7 The recovery of the velocity of the potential evoked by the test stimulus at the end of the absolutely refractory period as a funct on of the d stance from the point of stim ulation

The arrival times of the potentials evoked by the test stimuli were nor malized to the average velocity of the conditioning potential (2 4 m/sec full line)

d) *Absolutely refractory period and conduction velocity as a function of temperature*

The refractory period varied more with temperature than the conductio velocity of the conditioning potential Q_{10} was 2 0 and 1 6 respectively (14-25 C Table II)

2) *The period of recovery*

a) *Supernormal phase*

During the relatively refractory period the threshold of the potentia ev by the test stimulus decreased gradually and reached the threshold of conditioning potential at 1 8 times the absolutely refractory period W further increase in time interval between the stimuli of the pair the th to the test stimulus decreased it was 10 per cent below the threshold conditioning stimulus at three times the absolutely refractory period a from this minimum to reach normal at 35 msec mirroring the time diminution of the negati e afterpotential Thus the supern rmal ph about eight times as long as the absolutely refractory period (

Table I Conduction velocity (V) of the potential evoked by the conditioning and test stimulus at different temperatures

°C	No of fibres	Potential evoked by the		
		Conditioning stimulus V (m per sec)	Test stimulus V (m per sec)	$\frac{V_t}{V_{dist.}}$ (per cent)
14	6	1.7 ± 0.07	0.9	54 ± 3
20	6	2.3 ± 0.07	1.3	56 ± 1
25	14	2.8 ± 0.08	1.8	65 ± 1

Distance of the proximal and of the distal recording electrodes 0.4—1 mm and 4.5—6 mm from the stimulating electrode

Table II Temperature dependence of the absolutely refractory period and the conduction velocity

°C	No of fibres	Absolutely refractory period (m per sec)	Q (14—25°)	No of fibres	Velocity of the conditioning potential (m per sec)	Q_{10} (14—25°)
14	11	4.8 ± 0.11	2.0 ± 0.06	12	1.7 ± 0.03	1.6 ± 0.04
20	15	3.1 ± 0.10		21	2.3 ± 0.06	
25	30	2.2 ± 0.05		39	2.8 ± 0.05	

b) *The recovery in amplitude of the potential evoked by the test stimulus*

The amplitude of the potential evoked by the test stimulus had recovered from the 35 per cent reduction at the end of the absolutely refractory period when the interval between the stimuli was 2.5 times the absolutely refractory period (Figs 9A and 10). Thus the amplitude of the second potential took longer to recover than did its threshold (at 1.8 times the refractory period Fig 8). During the supernormal phase of excitability and conduction velocity the amplitude was not increased above that of the conditioning potential.

Recorded at about 5 mm from the stimulating electrode the amplitude of the potential evoked by the test stimulus at the end of the absolutely refractory period was 10 ± 0.3 per cent lower than that of the conditioning potential (p. 13). When the interval between stimuli was 1.1 times the absolutely refractory period the second potential was 12 ± 0.7 per cent lower than the conditioning potential (Fig 9A). At this time interval the second potential arrived earlier after the test stimulus than at the absolutely refractory period

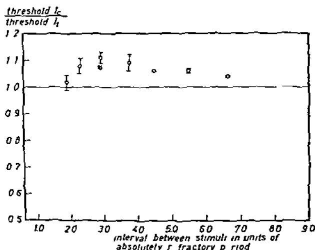


Fig 8 The supernormal phase of excitability in the relatively refractory period (14 C) Ordinate Ratio between threshold currents of the cond tioning (I) and the test stimulus (I_t)

The vertical bars indicate the standard error of the mean and the figures below the po nts the number of fibres examined

allowing less time for the amplitude to recover The earlier arrival is due to the shorter rise time of the action potential and to a recovery of conduction velocity at 1 1 times the absolutely refractory period as compared with the end of the absolutely refractory period

c) *The recovery in conduction velocity*

The recovery in conduction velocity in the relatively refractory period depended on the position of the recording electrode and on the temperature At 20 C with electrodes at 0 3 and 3 mm from the stimulating electrode the two potentials again reached equal velocity when the interval between the two stimuli was 3 times the absolutely refractory period with electrodes at 1 and 7 mm from the stimulating electrode the velocities were equal with an interval of 1 8 times the absolutely refractory period (20 C)

A supernormal phase of conduction velocity has been recorded in the late refractory period of myelinated frog nerve (22 C) (GRAHAM 1934) of papillary muscle (PILLAT and HEISTRACHER 1960) and in human skeletal muscle (FARMER *et al* 1960) In our experiments on fibres of frog muscle a supernormal phase was observed at 25 C At this temperature the velocity had recovered with intervals between the stimuli of 1 5 times the absolutely refractory period and was 10 per cent faster than that of the conditioning potential at 2—3 times the absolutely refractory period (Fig 9B) The total duration of the supernormal phase was not determined At 20 C the supernormality was less pronounced and at 14 C there was none with intervals between the stimuli up to three times the absolutely refractory period when supernormality of the excitabil·· was most pronounced

[illegible]

d) [illegible] of [illegible]

Repolarization of the conducting potential varied with temperature: the [illegible] at high than at low temperature [illegible] and [illegible]. [illegible] the negative afterpotential [illegible] the end of the rapid phase of repolarization was 2.5 [illegible] 0.5 mV at 2[illegible] C [illegible] 12 [illegible] 2 mV at 14 C (Fig. 11).

In the potential evoked at the end of the absolutely refractory period the slopes of the rapid phase of repolarization and of the negative afterpotential

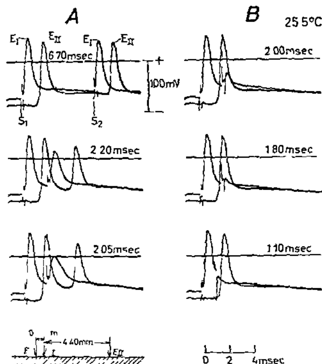


Fig 10 The potential evoked by the conditioning (S_1) and by the test stimulus (S) recorded simultaneously by an el ctrode at 0 55 mm (E_I) and 4 95 mm (E_{II}) from the st mulat ng electrode (E) In B S no longer evoked a propagated response

differed less than in the conditioning potential (Fig 4 and 10) At its onset the negative afterpotential was about 5—10 mV higher than in the condition ing potential

At about eight times the absolutely refractory period repolarization of the conditioning potential was almost complete the remaining depolarization being only 2 ± 0 4 mV (n = 18 14 C) When the interval between stimuli was two to three times the absolutely refractory period the negative afterpoten tial was of the same amplitude in the conditioning potential and in the poten tial evoked by the test stimulus

The absolutely refractory period terminated at the onset of the negative afterpotential (Fig 10) recovery in excitability amplitude and conduction velocity of the potential evoked by the test stimulus occurred while the mem- brane potential did not increase by more than 3 mV (14 and 25 C)

3 *The local response of the potential e oked by the test stimulus*

Immediately before the end of the absolutely refractory period when the test stimulus just failed to evoke a propagated potential the local response was 60 ± 5 mV recorded 0 25—0 6 mm from the stimulating electrode in 15

fibres (15° 20° and 25° C resting potential 81 mV) a higher level of depolarization than required to initiate the conditioning potential (40 mV FATT and KATZ 1951, JENERICK 1956) A similar difference has been described in crab nerve (HODGKIN 1938) When the test stimulus was near threshold the local response which just failed to initiate a propagated response was 40 ± 3 mV (6 fibres resting potential 84 mV) i e the same as that which just initiates a conditioning potential When a propagated potential was just elicited by a test stimulus near threshold the hump in the rising phase indicating the transition from initial depolarization was more pronounced than in a conditioning potential evoked by a threshold stimulus As the test stimulus approached the conditioning stimulus the amplitude of the local response decreased and it was *difficult to decide whether it was present at all A local response could* be discriminated with certainty at a time interval of *70—80* per cent of the absolutely refractory period of the propagated response

Discussion

After passage of an action potential evoked by direct stimulation of single skeletal muscle fibres the amplitude the rate of rise the conduction velocity of the second evoked action potential and the excitability of the fibre were diminished No propagated action potential could be evoked by supramaximal stimuli for 2 msec after the conditioning stimulus at 25 C for 4 5 msec at 14 C i e at transition from the spike to the negative afterpotential As the interval between the pair of stimuli lengthened excitability recovered first then the amplitude rate of rise and conduction velocity of the action potential *Complete recovery took place within a time interval in which the membrane* potential changed at most 3 mV

The potential evoked by the test stimulus

By intracellular recording in the single fibre the reduction in amplitude and in rate of rise of the potential evoked by the test stimulus depended on the distance of the recording electrode from the stimulating electrode At a distance of 0 6 mm the decrease in amplitude was 35 per cent and in rate of rise 60 per cent at the end of the absolutely refractory period at a distance of 4 mm the amplitude *differed by only 10 per cent and the rate of rise was nearly normalized*

By extracellular recording in fascicles of whole muscle the amplitude of the potential evoked just after the end of the absolutely refractory period was less than 25 per cent of that of the conditioning potential (LASER and STARK 1958) The extracellular potential is roughly proportional to the second derivative of the membrane potential i e roughly proportional to the amplitude of the intracellular potential and inversely proportional to the square of its rise time With a reduction of the amplitude by 35 per cent and of rate of rise by 60 per cent the amplitude of the extracellularly recorded potential is reduced by 75 per cent In the experiments of *(LASER and STARK no information is*

given as to the recording distance from their recordings it seems to be of the order of 4 mm assuming a conduction velocity of 4 m/sec Therefore the marked decrease in amplitude observed by GLASER and STARK reflects not only changes in the individual fibres but also *a loss in responding fibres with decreasing* time intervals between the stimuli The fact that FARMER *et al* (1960) by extra cellular recording in small bundles of human muscle found only a 10 per cent decrease in amplitude of the potential evoked by the test stimulus is consistent with the recovery of rise time and amplitude in the single fibre at distances larger than about 10 mm from the point of stimulation

That the test potential of the single fibre recovers from 65 to 90 per cent of full amplitude in the course of its passage to about 4 mm from the point of stimulation is consistent with the diminished conduction velocity which delays its arrival 1 5 times In fact when the time between the pair of stimuli is 1 5 times the absolutely refractory period the test potential recorded near the point of stimulation is 90 per cent of full amplitude

Conduction velocity

When conduction velocity is determined it must be considered that the position of the stimulating electrode may not coincide with the point of initiation of the impulse and that the latter may move with different strength of the stimulus Therefore it is essential to record from at least two points of the fibre both situated outside the region in which the impulse is initiated With extracellular stimulation of whole nerve the point of initiation of the nerve impulse did not coincide with the cathode but lay some 3 mm from it extra polarly (RUSHTON 1949) Similarly in small bundles of human muscle fibres arrival times of the *action potentials extrapolated to zero time indicated that* stimulation was initiated up to 8 mm proximally or distally from the bipolar stimulating electrode (BUCHTHAL GULD and ROSENFALCK 1955) With *intra cellular* stimulation of a single muscle fibre with intensities 1 5 to 2 times threshold the point of stimulation nearly coincides with the position of the stimulating anode

The question arises how the potential evoked by the test stimulus at the end of the absolutely refractory period is conducted from the stimulating electrode to the distant *recording electrode Propagation may proceed over a distance* of 1—2 mm without overshoot (Fig 5B) At distances of 2 mm or less KUFFLER (1942) reported evidence of conduction with decrement at the end of the absolutely refractory period He used stimulation of the muscle fibre through the nerve as test stimulus and found small spikes propagated not further than 2 mm from the end plate In heart muscle (KAO and HOFFMAN 1958) the amplitude of the potential evoked by the test stimulus after short time interval was greater near the stimulating cathode than at 2 mm from it In our *experiments in which the muscle fibre was stimulated directly there*

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course as the return of excitability from supernormality to normal However there is no direct relation between the degree of early recovery and the restitution of the membrane potential excitability action potential and conduction velocity recovered at a time when the membrane potential was practically constant Moreover metabolic poisons (dinitrophenol and azide) may diminish or abolish the afterpotential without affecting the increase in membrane conductance associated with activity (PERSSON 1960) As in Purkinje fibres of heart muscle (WEIDMANN 1955) supernormality can be explained by a more complete recovery of the threshold potential than of the membrane potential

Non propagated responses occur after the passage of an action potential when the fibre is still unable to produce a conducted response In skeletal muscle fibres as in the squid axon (HODGKIN and HUXLEY 1952) there was a time interval in which a local response after a supramaximal test stimulus could not be discriminated Whether this is a true refractory state or whether a local response is masked by the increase in membrane conductance in the falling phase of the action potential is undecided

Summary

Refractory period and conduction velocity of transmembrane potentials were determined in single frog muscle fibres by intracellular stimulation and recording The absolutely refractory period was 2 3 and 4 5 msec at 25 20 and 14 C (Fig 3B) and was obtained when the test current was 1 5 times the threshold current The conduction velocity recorded between two electrodes separated by a mean distance of 5 mm was 2 8 2 3 and 1 7 m per sec at 25 20 and 14 C (Fig 6B) The refractory period varied more with temperature than the conduction velocity

The properties of the potential evoked by the test stimulus at the end of the absolutely refractory period differed according to the distance from the stimulating electrode at which the potential was recorded *At 0 5 mm* the rate of rise was diminished about 60 per cent and the amplitude was 35 per cent lower than the amplitude of the conditioning potential *At 4—6 mm* from the stimulating electrode the rise time was reduced by about 30 per cent and the amplitude by only 10 per cent There was no evidence of a conduction with decrement

The differences in the properties of the potentials recorded at different distances were due to a 40 per cent lower conduction velocity of the potentials evoked by the test stimulus This was the main cause of the late arrival of the potential delayed firing causing at most 10 per cent of the total delay The velocity approached normal at about 5 mm from the point of stimulation (Fig 7)

Conduction to 1—2 mm might occur with amplitudes of 70 mV 20 per cent below the potential of the resting membrane (Fig 5 B)

Recovery of excitability occurred at 1 8 times the absolutely refractory period it was 10 per cent supernormal at three times and normal at about eight times the absolutely refractory period (Fig 8 14° C) At this time interval the membrane potential was almost restored *Recovery of amplitude* occurred at 2 5 times the absolutely refractory period *Recovery of conduction velocity* was obtained at 1 8—3 times the absolutely refractory period (20 C) with the lowest value when recorded at the shortest distance from the point of stimulation At time intervals of 2—3 times the absolutely refractory period and at 25 C the conduction velocity was 10 per cent supernormal (Fig 9B) Recovery in excitability amplitude and conduction velocity of the potential evoked by the test stimulus occurred while the membrane potential did not recover more than about 3 mV

At the absolutely refractory period the level of depolarization required to initiate a propagated response had increased from 40 mV to more than 60 mV (recorded 0 25—0 6 mm from the stimulating electrode) A local response appeared at a time interval of 70—80 per cent of the absolutely refractory period of the propagated response

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Note added in proof

In cardiac muscle VAN DAM R T E. N MOORE and B F HOFFMAN (Amer J Physiol 1963 *04* 1133—1144) have recently reported an apparent supernormal phase in conduc tion velocity in the relat vely refractory period due to a shift in the site of origin of the

the abundance of large mitochondria in tissues known to have a high aerob metabolism that the functioning of the *rete* does not involve a high expenditure of energy

A *rete* whose main property is diffusion exchange between arterial and venous blood will serve very different functions depending on the solubility of the diffusible gas in the two blood streams If the solubility is the same the *rete* will constitute a barrier not only for gas to escape the bladder but also for gas to enter it If on the other hand the solubility of the gas is decreased (i e gas tension increased) as the blood circulates the bladder then gas will diffuse from venous to arterial blood in the *rete* The arterial blood is therefore enriched in gas and when it returns to the *rete* as venous blood the gas tension will again be increased and drive more gas into the arterial blood By this mechanism which is termed hairpin counter current diffusion multiplication high gas pressures will be built up in the blood at the bladder pole of the *rete* allowing gas to enter the bladder by diffusion The limit of performance of such a system will be determined mainly by the solubility reduction the diffusivity of the *rete* tissue the length of the *rete* and the velocity of blood flow (KUHN and KUHN 1961) It is also worth notice that a *rete* working in this way will be a barrier for gas loss from the bladder while at the same time it will allow gas to enter the bladder through the *rete*

Theories on gas concentration The combination of O_2 with fish blood from all investigated fishes known to have a functioning swimbladder suffers a decreased O_2 affinity on addition of acid (Bohr effect) and some also a reduced O_2 capacity (Root effect) (ROOT 1931 SCHOLANDER and VAN DAM 1954 MANWELL 1960) This property of fish blood immediately suggests that an acid may be deposited into the venous blood of the gas gland creating a primary gradient which will be multiplied by the *rete* to higher pressures

This mechanism of gas concentration was in principle proposed by HALDANE (1922 p 215) and later extended by HALL (1924) and JACOBS (1930) KOCH (1934) realized that an acid electrolyte added to the blood would effect not only O_2 bound to hemoglobin but also cause a reduced solubility of all gases present in the blood and thus it might explain concentration of inert gases as well SCHOLANDER (1954) developed simple equations for the quantitative potentialities of the *rete* working as a multiplier to concentrate gases in the swimbladder KUHN and KUHN (1961) have later carried out a more detailed mathematical treatment of the same Both these workers conclude that if some acid substance is added to the venous blood stream in sufficient quantity to reduce the amount of O_2 bound to hemoglobin by 10 % and the physical solubility with 1 % then the *rete* is able to create pressures which actually exceed those recorded from the swimbladder of deep sea fishes

SCHOLANDER (1954) however came to the conclusion that the substance which is to induce the primary gradient in O_2 tension could not be an acid This was based on the observation of SCHOLANDER and VAN DAM (1954) that

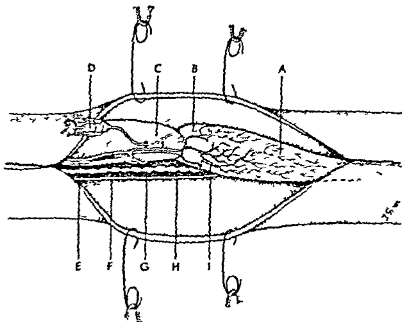


Fig 1 Shows a full scale drawing of the swimbladder of an eel A modified cannula is inserted into the secretory bladder which is separated from the reabsorbent bladder Intestines and gonads removed
A secretory bladder B = *rete* C = reabsorbent bladder D = modified cannula E = *pre re e* artery F = *post-rete* vein. G = dorsal artery H = dorsal vein I = *post rete* artery and *pre rete* vein

not even pH down to 6 0 was able to dissociate the oxyhemoglobin of some fishes against the O_2 pressures actually recorded in their swimbladders (SCHOLANDER and VAN DAM 1953) The validity of this conclusion will be reconsidered later in this paper

WITTENBERG and WITTENBERG (1961) investigated gas concentration in toadfish kept in water containing CO in addition to O_2 They found that CO was concentrated about 5 times more than O_2 when compared to the partial pressure of these gases in the water The authors argue that their results exclude a concentrating mechanism based on acid dissociation KUHN and KUHN 1961 however claim that their counter current multiplier model explains the results of WITTENBERG and WITTENBERG (1961) very satisfactorily

WITTENBERG and WITTENBERG (1961) suggest that the initial concentration gradient is brought about not by acid but by active secretion of O_2 from the venous to the arterial capillaries of the *rete* This modification implies that the superb diffusion properties of the *rete* are not used to build high pressures but instead to counteract the gradient created by the alleged active transport

There is experimental evidence indicating that the venous blood of the gas gland has a lower pH than the arterial thus indirectly lending support to the acid counter current multiplication theory HALL (1924) found that the pH

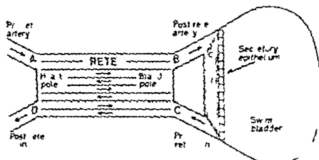


Fig 2 Shows a simplified drawing of the gas gland with *rete* and bladder The figure also gives the anatomical designations which are used in the text

of a dialysate of a homogenate of active glands of the yellow perch (*Perca flavescens*) was 6 4 against 7 1 for a passive one JACOBS (1932) found very high CO_2 values in freshly deposited gas from *Perca fluviatilis* BALL, STRITTMATTER and COOPER (1955) found that gas gland tissue from several species converts most of its glucose to lactic acid even in an atmosphere of O_2 HOGBEN (1958) measured the pH in two chambers initially filled with identical fluid but separated by the epithelium from the bladder of codfish The pH of the solution of the serosal side fell markedly whereas the pH in the other chamber remained unchanged FANGE (1953) found higher lactate content in homogenates of active glands than of passive ones in codfish Recently KUHN MOSER and KUHN (1962) and STEEN (1962) reported that in the active bladder of the eel the lactic acid content of the blood leaving the *rete* was up to 60 mg % higher than in the blood entering it

The problem There is little doubt about the potentialities of the gas gland to concentrate gases by counter current multiplication of a gradient created by the presence of a larger concentration of an acid electrolyte in the venous branch of the *rete* than in the arterial The aim of this investigation was to produce evidence to show if this possibility is in fact the operating mechanism of gas secretion and if so to discuss if it will suffice to explain gas secretion also in the most extreme cases of deep sea fishes

Experimental approach An answer to these problems has been sought through measurements of the gas composition in the bladder and of the blood from arterial and venous vessels at both poles of the *rete* during activity This has been accomplished by measurements of the content in the blood of O CO, and lactic acid and of blood pH By comparing these data with O_2 and CO_2 equilibrium curves the tension of these gases in the blood was obtained In connection to these main experiments the influence upon the rate of O_2 deposition of the O_2 pressure in the bladder was investigated

Experimental animal The eel was found to be a suitable animal for these experiments It deposits gas willingly even when lying in the air with the bladder

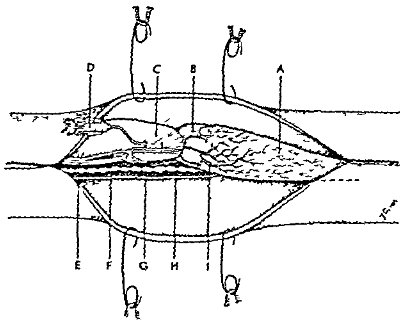


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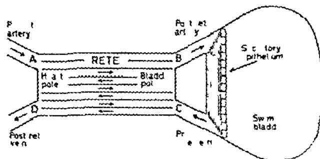


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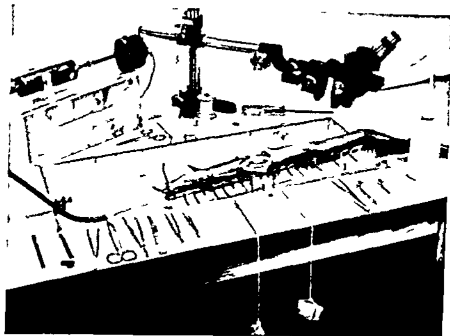


Fig 3 This shows the experimental arrangement in unusually good order The eel is fastened in the rack and supplied with water The abdomen is opened and the bladder is exposed The sample storer is shown to the upper left

exposed Moreover the anatomy of the swimbladder is very favourable (Fig 1) By placing a ligature around the duct connecting the secretory bladder (A) with the reabsorbent bladder (C) their contents can be separated and gas deposition can be studied undisturbed by reabsorption The bladders receive blood from the swimbladder artery (E) a branch of which supplies each *rete* from the heart pole (Fig 1 and 2) At the bladder pole of the *rete* the capillaries re unite in 3 to 5 *post rete* arteries These run parallel to the same number of *pre rete* veins returning blood from the bladder to the *rete* At the heart pole of the *rete* the two *post rete* veins unite and the blood enters the hepatic portal vein Thus all the blood which circulates in the secretory bladder wall goes through the *retia* The dimensions of these arteries and veins are large enough to permit blood sampling with thin hypodermic needles

Material

Experiments were performed on female specimens of the eel *Anguilla vulgaris* These were bought at the fish market in Oslo The animals were kept without food in fresh water tanks

Preparation of the eels for experiments The unanesthetized eel was fastened in a rack as shown in Fig 3 and supplied with fresh water through a glass tube tied into its mouth The fish was opened ventrally and the bladder freed from connective tissue exposing

its vascular supply quite clearly. Circulating red cells could be observed through the transparent vessel walls with the aid of a microscope.

A hypodermic needle modified as shown in Fig. 1 was inserted into the secretory bladder through a small incision in the reabsorbent bladder. A ligature around the duct connecting the two bladders served both to fasten the needle and to separate the bladders. The contents of the secretory bladder were anaerobically reached by puncturing the rubber stopper closing the head of the needle. This preparation permitted therefore measurements on the unopened separated secretory bladder with blood supply through one artery and one vein only (cf. Fig. 1).

In many experiments gas deposition started as soon as the bladder had been emptied. Intramuscular injection of 1 ml of a 0.1 % aqueous solution of yohimbine increased the activity which seemed to be further enhanced by applying pressure on the bladder, e.g. by tying bands moderately tight around the fish. The secreting bladder was distinguished by good blood circulation and well oxygenated arterial blood in contrast to the passive bladder where circulation was absent and where the blood was dark, poorly oxygenated and with a low pH. Lack of secretion was therefore not taken as representative for the normal passive gland, but rather as an indication that the experimental situation had resulted in anoxia. No passive glands were found without these anoxic tendencies.

Methods

Analytical methods. The content of O_2 in 40 μl of blood was measured by the method of ROUGHTON and SCHOLANDER (1943) with the modifications prescribed for fish blood by SCHOLANDER and VAN DAM (1956). Duplicate analyses on eel blood agreed within 0.4 vol. %. The content of CO_2 in about 13 μl of blood was measured by the method of SCHOLANDER and ROUGHTON (1943). Duplicate analyses usually agreed within 0.7 vol.%. Gas-samples were analysed with the water analyzer of SCHOLANDER *et al.* (1955). Duplicate analyses agreed within 0.3 %. The pH of 20 μl blood was measured with a Metrohm pH meter equipped with a capillary micro blood electrode. The instrument was checked prior to each measurement against a buffered solution of pH 7.12. The pH of duplicate blood samples agreed within 0.02 units. The content of lactate was measured by a micro diffusion method (CONWAY 1962) modified by SCHOLANDER and BRADSTREET (1962) to allow analyses on samples no larger than 5 mg. Duplicate analyses on blood samples agreed within 6 mg %. The method is not specific for lactic acid, but will determine any compound which gives acetaldehyde on oxidation with $CeSO_4$ at very low pH. It is unlikely, however, that any such substances are present in sufficient quantities to impart a serious uncertainty.

Hematocrit was determined within 1 % in a conventional hematocrit tube.

Derived values. The tensions of O_2 and CO_2 in the blood were estimated from the measured gas contents, pH values and hematocrit by the use of previously determined equilibrium curves (STEEN 1963 a). The measured O_2 content was first normalized to hematocrit 40 % in the following way. The measured O_2 content was assumed proportional to the actual hematocrit, and the O_2 tension was estimated by comparison to the O_2 equilibrium curve. The obtained O_2 tension was used to get an approximate value of the dissolved O_2 (α for O_2 = 3.0). This was subtracted from the measured O_2 content. The remaining was considered proportional to the measured hematocrit whereby it was normalized to a hematocrit of 40 %. The sum of the dissolved O_2 and the normalized hemoglobin bound O_2 represented then the O_2 content of blood which had been found had the hematocrit been 40 %. This procedure induced an error of at most 0.3 vol. %. A consideration of the effect of the experimental procedure on the accuracy of the estimated O_2 tension will be found at the end of this chapter.

The blood flow through the bladder in ml/min was calculated by dividing 1/60th of the amount of O_2 deposited in one hour with the difference in O_2 content in the *pre rete* artery and the *post rete* vein The values are only approximate since the method assumes that the same difference in O_2 content was present during the hour of gas deposition

The velocity of the blood in cm/min through the *rete* was roughly estimated by dividing the flow with the average cross sectional area for arterial and venous capillaries in the *rete* of the eel (KROGH 1929) The variable size of the *retia* from one eel to another of course induced a considerable error in the values obtained

The rate of gas deposition was determined by measuring changes in volume and composition of the bladder gas The volume was measured by drawing the gas in the bladder into one or more 1 ml tuberculine syringes recording the volume and reintroducing the gas The composition of the gas was measured by analyses of a 0 05 ml sample This procedure gave the rate of gas deposition of various gases within 0 03 ml For further details confer STEEN (1963 a)

Blood samples measuring up to 0 1 ml were obtained by puncturing the appropriate vessels with a modified hypodermic needle fitted to a blood pipette (*cf* STEEN 1963 b) Care was taken that air was not trapped at this point during sampling Prior to use a drop of heparin was run through the pipette which was then dried by suction During sampling the pipette was wrapped in wetted gauze and handled with a wooden cloth pin to reduce the extent of supersaturation Despite this some bubbles always formed as blood from *post rete* artery filled the pipette In most cases this cavitation resulted in very few and barely visible bubbles, which easily followed the blood It should be stressed that when the blood samples were later transferred to the blood gas analyzers care was taken that a proportional amount of these bubbles followed it The collected samples were immediately placed in a sample store (Fig 3) which was cooled and continuously rotated 180 back and forth to reduce sedimentation The blood samples were treated as follows 20 μl blood was deposited in a clean hydrophobic porcelain cup and immediately sucked into the capillary pH electrode The blood was in contact with air for no more than 15 sec pH values obtained on blood treated in this way checked accurately with the values obtained when the blood was handled strictly anaerobically Subsequent to pH measurement the blood was returned to the cup and sucked into the original pipette again separated from the rest of the original sample by an air meniscus The pH electrode was carefully rinsed with distilled water between each sample Two samples from each pipette were analysed for lactate Small glass vials had been prepa rated with 1 ml 5 % trichloracetic acid in each The vials were weighed before and after the blood had been added When the blood had been added to the vial it was vigorously beaten to allow the necessary extraction of the lactate The vials were closed with rubber stoppers and analysed the following morning The rest of the blood was used for O_2 and CO_2 analysis The CO_2 analyser was first charged and the procedure carried out until the CO_2 had been extracted and the syringe set aside for temperature equi libration At this time the O_2 analyses were started The sequence of the analyses was always the same first the pH measurements then preparation of lactate samples and finally CO_2 and O_2 analyses With this procedure time induced changes which certainly occur should be about the same in all samples

Validity of data on O_2 and CO_2 tension The accuracy of the data obtained by the use of O_2 and CO_2 equilibrium curves to estimate gas tensions warrant special attention The equilibrium curves themselves suffer a certain uncertainty in all parameters com pared to the "true values This has little importance however as long as 1) the same set of curves were used to obtain tensions from all experiments and 2) the results are compared only to those obtained during the same experiments

Table I Shows the rate of deposition of O_2 and N_2 in experiments where the partial pressure of gases in the bladder varied within 1 atm

Eel no	Gas composition				Gas deposition	
	Initial		Final		ml/hour	
	O	N	O	N	O	N
1	36	59	57	39	0.37	0.013
1	5	93	31	69	0.37	0
1	3	95	55	41	0.37	0.02
2	2	89	23	74	0.13	−0.02
2	97	5	92	6	0.10	0.02
3	4	93	41	55	0.35	0.03
3	93	5	90	5	0.36	0.03
3	4	94	37	59	0.36	−0.02

Of importance on the other hand are the factors which influence the reliability of the estimated O_2 content and pH The accuracy in the O_2 content is mostly influenced by hematocrit and analytical error The hematocrit induces an error of ± 0.2 vol % Under experimental conditions when the samples were stored for some time, the analytical accuracy was less than that given in the chapter on the methods Duplicate pH measurements agreed within 0.04 units O_2 measurements within 0.6 vol % and CO_2 within 1.2 vol % The procedure did not seem to induce any tendency in the results towards higher or lower values The reduced accuracy is probably due to a combination of slight sedimentation respiration with loss of O_2 and increase in CO_2 and/or lactic acid gas exchange with the air at the upper end of the blood pipette and foam formation in some cases

Only in experiment G (Fig 5) did the volume of foam formed in the blood sample from the *post rete* artery exceed 1 μl In this case 5 μl formed in 100 μl blood 3 μl of this gas was analysed and showed 16 % CO_2, 67 % O_2 and 17 % N_2 This means a CO_2 loss of 0.8 vol % Blood from the *post rete* artery had a pH between 7.0 and 7.4 A CO_2 loss of 1 vol % will increase the pH with at most 0.05 units at a pH of 7.4 and 0.01 unit at pH 7.0 CO_2 loss by cavitation does therefore not reduce the accuracy of pH measurement significantly (except in exp G Fig 5)

Considering all the uncertainties involved in determining the O_2 content and pH the estimated O_2 tension is probably accurate within $\pm$ 0.15 atm in the pressure range where hemoglobin is saturated At lower pressures the accuracy will of course be much better e g at pH 7.5 and half saturation the O_2 tension is accurate within $\pm$ 0.01 atm The CO_2 tensions are probably accurate within $\pm$ 0.01 atm

Experiments

All the experiments were performed on the preparation of the secretory bladder which has already been described The prevalent temperature of the eel and its water supply ranged from 18 to 20 °C

1) *The influence of pO_2 upon the rate of O_2 deposition* was investigated in the following way When a preparation had displayed a constant rate of O_2 deposition for a few hours it was emptied and filled with a known mixture of O_2 and N_2 Two min later the gas was removed and replaced by 1 ml of the same gas mixture 0.05 ml of this was withdrawn

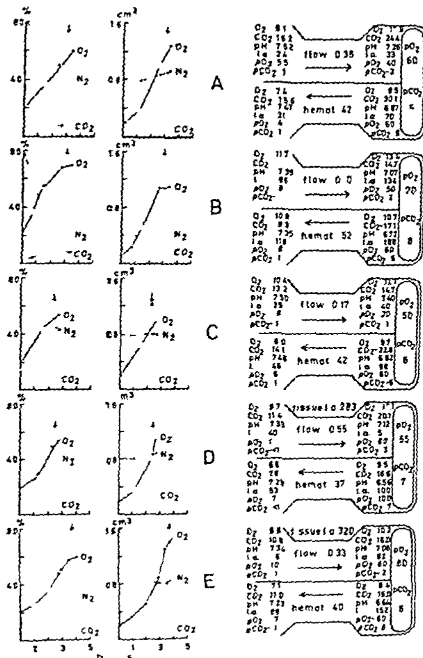

A
B
C
D
E
flow 0 35
hemat 42
flow 0 0
hemat 52
flow 0 17
hemat 42
flow 0 55
hemat 37
flow 0 33
hemat 40

Fig 4

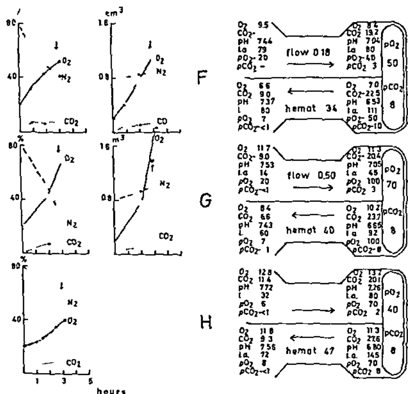


Fig 5

Figs 4 and 5 These show the variat on in the concentration and volume of gases in the bladder during gas deposit on. At the poi it ind cated by an arrow blood samples we e taken The results of the analysis of these samples are shown on the s mplified *rete* to the right. The tens ons of O and CO have been calculated and the values are written in italics The hematocrit and blood flow is also included for each experiment The content of O and CO in the blood is given n vol the lactic ac d content in mg blood flow in ml/min hematocrit n % red cells and pO_2 and pCO as of o ie atm

and analysed immediately The first sample was used to adjust the pO_2 of the bladder tissue to the new gas The rate of deposition was then measured for one hour The procedure was repeated with different mixtures of O_2 and N_2 (Table I) Similar experiments were conducted when the bladder artery and vein had been tied off

2) *The mechanism of gas secretion* was studied a follows The separated bladder was emptied and then repeatedly filled and emptied with air a measured volume of which was finally injected At certain time intervals thereafter the content of O_2, N_2 and CO_2 in the bladder was measured When the bladder had shown unmistakable signs of activity for some hours (see Fig 4 and 5) the blood sampling was started During sampling a rubber tube was attached to the end of the pipette so that suction could be applied if a clot formed in the needle or if the blood pressure became too low to fill the pipette This was almost exclusively used in sampling from the *post rete* vein The needle was small enough to allow blood to flow in the vessel during sampling although flow conditions were certainly not normal

The functioning of the gas gland is undoubtedly influenced by changes of the flow or leakage of blood from the system I sought to minimize disturbances of this sort by taking samples in the following sequence (cf Fig 1 and 2) the *pre rete* vein of one *rete* 2) the *post rete* artery of the other *rete* 3) the *post rete* vein and finally 4) the *pre rete* artery In experiments B and E the sequence was 3 1, 2 4 Two minutes were allowed between samples so that the disturbances of the sampling could be normalized Each sampling lasted from 2 to 5 min and about 20 min elapsed from starting the first to finishing the last sample Immediately after the last sample had been taken the volume and composition of the bladder gas was recorded and a blood sample taken for hematocrit In two experiments the bladder stripped of most of its connective tissue and the *retia* and gently squeezed to remove blood was weighed and crushed thoroughly in a measured volume of trichloracetic acid for later determination of lactate The elapsed time between removal of the bladder and its immersion in the acid never exceeded 3 min

Results

In Table I the rate of gas deposition is compared to the gas composition in the bladder It is apparent that the rate of O_2 deposition is unaffected of a change in O_2 pressure within 1 atm The N_2 deposition is too low to allow any further conclusions When the vascular supply to the bladder was tied off no movement of gas could be detected during 3 hours

The results of the measurements of the changes in gas composition in the bladder during activity are shown in Fig 4 and 5 The content of O_2 in the bladder increases the N_2 content remains rather constant while the CO_2 content increases in such a way that CO_2 always constitutes some 6 to 8 % of the gas The gas which is deposited during activity consist therefore of O_2 admixed with some 7 % CO_2 Only in experiments A and G does the amount of N_2 show a definite increase The N_2 tension was in all cases, however lower in the bladder than in the water which means that N_2 is in no case concentrated

The time of blood sampling is indicated for each experiment in Fig 4 and 5 As seen from the curves the rate of O_2 deposited was rather constant at this point, but in some cases it decreased during sampling This is not due to blood loss as the sampled blood volume represents only 1/10th to 1/20 of the total blood flow In some cases however, sampling seemed to induce contraction of the bladder sometimes only locally and during a shorter period This always resulted in a decreased blood flow These irregularities together with the disturbances by removing blood and changing the flow during sampling influence the results in an unpredictable manner which demands caution in comparison of quantitative results both within one experiment and especially when comparing different experiments Particular stress will therefore be laid upon the general trends in the results

The results for the blood analyses are shown in Fig 4 and 5 There is a fair correlation between the measured pH values and those which are obtained when the pH is extrapolated from CO_2 equilibrium curves (STEEN 1963 a) using the values for CO_2 and lactate content measured in each sample Since

Table II Shows a quantitative comparison between two models for gas concentration and the values measured in the present investigation The latter pertain to the situation at the heart pole of the rete

	mM electrolyte in *post rete* vein above that in *pre rete* art	% reduction in physical solubility	Flow (ml/min)	Velocity of flow (cm/min)	% reduction of O capacity	Max N pressure (atm)	Max O pressure (atm)
SCHOLANDER (1954)	—	1	1	(≈20)	≈30	26	4 000
KUHN and KUHN (1961)	20	1	0 6	12	10	26	2 000
	20	1	0 3	6	—	53	—
	10	0.5	0 3	6	—	26	—
Present work	3	—	0 1–0 5	3–10	10–35	—	—

the equilibrium curves were obtained on blood where pH was varied by CO_2 and lactic acid this indicates that the lactate content represents lactic acid

The composition of blood from arteries and veins at both poles of the *rete* of active glands displayed the consistent pattern illustrated in Fig 4 and 5 Blood enters *rete* with arterial O_2 content and a low CO_2 content The O_2 tension of this blood is below 0 2 atm and the CO_2 tension is below 0 01 atm During its passage through the *rete* the arterial blood is enriched in lactic acid and CO_2 the pH is reduced and the O_2 content remains rather constant The O_2 tension however increases and it is noteworthy that when the pO_2 in the bladder is high the O_2 content of the arterial blood may indeed increase as it passes the *rete* As the blood circulates through the epithelium of the bladder its lactic acid content is further increased while the pH and O_2 contents are reduced The O_2-tension of the blood from the *pre rete* vein is in most cases equal to or higher than the O_2 tension inside the bladder On passing the *rete* the venous blood looses some of its lactic acid the pH increases the CO_2 content decreases and the O_2 content as well as the O_2 tension are reduced The lactic acid content of the bladder tissue is at least twice that in the *pre rete* vein

It should be stressed that at the bladder pole of the *rete* the O_2 tension is generally higher in the vein than in the artery while at the heart pole it is lower in the vein than in the artery Apparently the direction of the tension gradient is reversed by the *rete*

The blood flow through the bladder varied between 0 1 and 0 6 ml/min (Fig 4 and 5) The velocity of the blood flow in the *rete* varied from 3 to 10 cm/min (Table II)

The cavitation of the blood from the *post rete* artery clearly indicates that the total gas tension in this blood must have been well above 1 atm Our knowledge about the conditions which facilitate cavitation in blood does not permit to conclude however, that the tension in the *post rete* artery was higher than in the *pre rete* vein although blood from the latter location did not cavitate

The lactic acid content in the *pre rete* vein is higher than the sum of the lactic acid content in the *post rete* vein and the *post rete* artery minus that in the *pre rete* artery This discrepancy may be due to conditions induced by the sampling procedure *e g* change in flow or reduction of the blood volume in one branch of the *rete* Both these artefacts certainly occur but no good ways have been found to determine their importance in this connection It was also possible that the first sampling induced a reduction in lactic acid production This was checked by lactic acid analyses in 3—4 samples taken from the *pre rete* vein 2 min apart No reduction or other systematic change could be determined, however The possibility also exists that some lactate is further metabolized or used in synthesis FANGE (1953) showed that the gas gland of codfish contains lactic acid dehydrogenase

Discussion

This investigation shows that during gas secretion in the swimbladder of the eel the lactic acid concentration is higher and the pH lower in the venous branch of the *rete* than in the arterial It was further established that the O_2 tension of the blood at the bladder pole increases as secretion proceeds and that the blood draining the secretory epithelium has an O_2-tension either equal to or higher than the pO_2 in the bladder The addition of lactic acid to the venous *rete* blood probably means that the total ionic concentration is increased whereby the solubility to gases in general will be decreased compared to that in the arterial blood

These observations strongly indicate that the active gas gland of the eel is endowed with the cardinal prerequisites for concentration of both O_2 and inert gases through a counter current multiplication of a primary gradient created by addition of lactic acid to the venous branch of the *rete* Considering the evidence for an increased acid content in active glands of other fishes (*cf* introduction) and the principal uniformity of the structure of the gas gland and the properties of blood from different fishes it seems possible that the same mechanism does also operate in other fishes

Behaviour of lactic acid and CO_2 during gas secretion HALDANE (1922) suggested that the venous blood of the *rete* communicated part of its CO_2 to the arterial blood JACOBS (1930) assumed that a substance which he thought was deposited into the blood followed the venous blood through *rete* but that CO_2 liberated by this substance diffused across the *rete* KUHN and KUHN (1961) assumed the *rete* to be completely impermeable to the added acid My results show that the situa

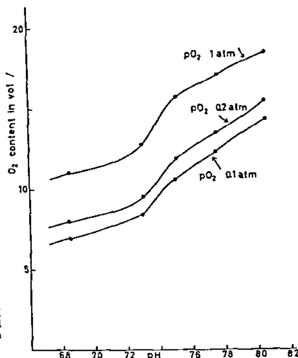


Fig 6 Shows the variation in O content of blood with varying pH and equilibrated at 0 1 0 2 and 1 0 atm O at 19 C Data from STEEN (1963 a)

tion in the eel is a mixture of these suggestions part of the lactic acid and CO_2 passes across the *rete* The CO_2 content is approximately the same in both blood streams making the CO_2-tension — due to the difference in pH — somewhat higher in the venous than in the arterial blood (Fig 4 and 5)

As mentioned above some of the lactic acid which enters the *rete* in the blood from the secreting bladder enters the arterial blood but some is also tracklessly lost This loss may possibly be due to some experimental artefact but more probably it may be due to lactate metabolism in the *rete* Since the loss entails a reduction in the acid gradient across the *rete* it may reflect one way in which the rate of gas deposition is regulated This assumption is strengthened by the fact that no such lactic acid loss was recorded in experiment G (Fig 5) which was also distinguished by the highest rate of O_2 deposition recorded It is tentatively assumed therefore that under maximum secretory work with a constant rate of lactic acid production the acid gradient from venous to arterial blood is the same at all points along the *rete*

The delayed response The short circuiting of acid across the *rete* will entail significant consequences for the efficiency of the multiplier These consequences are due to the fact that some time interval will necessarily elapse from acid is added to — or removed from — blood and until this is manifested as a changed O_2-tension In the further discussion this will be termed *delayed response*

We must assume that the O_2 capacity of the hemoglobin is determined by the pH inside the red cells When the acid content of the blood is changed this intracellular pH also is changed, but only after a delay caused by the time necessary for diffusion, for buffer systems to react and rearrange and for the reaction between hemoglobin and O_2 to occur (ROSKENBLECK and NIESEL 1962) The blood pH was always measured some 5—10 min after sampling During this time the intra and extracellular pH probably will have had time to read just The measured pH values will therefore not represent the intracellular pH at the time the sample was taken Generally the measured pH will be higher than the ' true pH in the blood which is losing acid and lower in blood which is gaining acid This means that the pH *measured* in the *post rete* artery is lower and in the *post rete* vein higher than the pH which dictated the O_2 tension at *the moment blood was sampled The delayed response* will therefore induce an error in the estimation of O_2 tensions by means of O_2 equilibrium curves In the blood from the *post rete* artery the pH is in the range 7 0 to 7 2 in the *post rete* vein it is from 7 3 to 7 6 Fig 6 shows that the O_2 capacity of eel blood is most sensitive towards pH changes in the range from 7 3 to 7 6 The delayed response will therefore induce the largest error in the estimation of O_2 tension in blood from the *post rete* vein And since the true pH will be lower than the measured for this blood the estimated O_2 tension will also be too low

It was mentioned earlier that the O_2 tension in the *post rete* vein apparently was lower than in the *pre rete* artery It seems justifiable to attribute this to an artefact caused by neglecting the delayed response in the estimation of the O_2 tension The O_2 tension in the *post rete* vein would have been equal to that in the *pre rete* artery had the pH in the former been 0 05 to 0 10 pH units lower The pH of the venous blood is increased about 0 7 units (Fig 4 and 5) as it passes the 7 mm long *rete* in 7 sec (Table I) A pH difference of 0 1 unit cor responds therefore to a delay of 1 sec between electrolyte change and readjust ment of O_2 tension In order to estimate the probability of a delay of this magnitude it is tempting to compare gas exchange in the *rete* with that in the lungs In the human lung the pH of the blood changes about 0 1 unit and each red cell is in estimate contact with the alveolar air for at least 0 3 sec in exercising subjects (ROUGHTON 1954) We may assume that the lungs are so developed that they allow the red cells just enough time in the lungs for the necessary *reactions to occur Considering that the human red cells are flat* disks about 8 μ in diameter while the eel red cells are bean haped with the smallest diameter 15 μ it seems fair to suggest that a much longer time would be needed for similar processes to occur in the eel blood A dependable judgement of this point must await direct measurements by physiochemical methods of the magnitude of the delayed response

In conclusion it seems probable that the O_2 tension gradient across the *rete* is caused partly by the actual acid gradient and partly by the delayed response Under steady state conditions with a constant lactic acid production

and a constant lactic acid concentration in the *pre rete* vein the flux of acid will not reduce the acid gradient across the *rete* Under such conditions therefore the acid flux will enhance the ability of the system to build high pressures above that which would have occurred had there been no such acid flux

The delayed response will probably be less important with regard to gradients in inert gases since these are physically dissolved and not like O_2 combined to a complicated physio-chemical system within a cell membrane

The rete The structure of the *rete* may also indicate properties of the system which are different from those assumed in the models The exchange of material in the *rete* takes place over very small distances and through complex cellular structures where the laws of diffusion may not suffice The structure of the barrier between the two blood streams shows an abundance of pinocytotic vesicles (FAWCETT and WITTENBERG 1959 DORN 1961) which may indicate that a one way transport of material is superimposed on the diffusion exchange Pinocytosis may also alter the diffusion properties of the barrier *e g* due to convection in the liquid inside the vesicles Furthermore the arterial capillary endothelium in some species is thicker than the venous with nuclei protruding into the lumen It appears also that the total cross sectional area of the arterial capillaries are smaller than that of the venous KROGH (1929) calculated based on the measurements of a fixed preparation of the *rete* from the eel that *the total* cross sectional area of the 118 000 arterial capillaries was only *half* of that of the 88 000 venous If this difference is present in the functioning gland it implies that the afferent blood flows twice as fast as the efferent a fact which should be considered in quantitative calculation on the efficiency of the gas gland

Comparison between models and experimental data It is evident from the foregoing discussion that even the most detailed models of counter current multipliers that have been presented represent merely a skeleton of the very complex and intricate process of gas concentration It seems clear however that the models represent the basic principle in gas secretion It is interesting therefore to compare some quantitative aspects of the models presented by SCHOLANDER (1954) and by KUHN and KUHN (1961) with experimental data from the present investigation Such a comparison is shown in Table II

It is evident that the assumed values coincide well with those actually measured This indicates that if the refinements which appear to occur in the multiplier system of the swimbladder have not reduced its efficiency relative to that of the model it should be able to build pressures of O_2 well above those actually measured The concentration of inert gases is more questionable A conclusion on this point must await measurements of the total ionic strength of the blood in the *rete*

The significance of CO_2 There is much evidence which indicate that the high CO_2 content of freshly secreted gas has functional significance COPELAND (1952) has shown that during activity the carbonic acid anhydrase activity decreases

in the gland plus *rete* while it increases in the blood This probably means that the enzyme is released into the blood during activity FANGE (1953) showed that the carbonic anhydrase activity is more concentrated in the secretory epithelium and *rete* than in the reabsorbent area and that inhibitors of this enzyme block gas secretion It seems reasonable that the function of carbonic anhydrase is to enhance CO_2 exchange both across the *rete* capillaries and across the secretory epithelium in the same way as it does in CO_2 exchange in the lungs

The high CO_2 content contributes to the lowered pH of blood from the bladder pole of the *rete* It has been shown earlier that a certain amount of CO added to blood will be more efficient in lowering the pH the more lactic acid is present beforehand (STEEN 1963 a) This means that even though there is no gradient in CO_2 content across the *rete* the CO_2 will cause a larger drop in pH in the venous than in the arterial branch of the *rete* due to the higher lactic acid content in the former

It was observed by FANGE (1953) that during activity the gas gland was covered by foam A similar observation was always noted in active eel bladders in the present study FANGE (1953) also demonstrated the presence of extra cellular cavities (secretory ducts) in the secretory epithelium of several species These probably represent gas cavities and their function may be to allow a rapid gas exchange between blood and bladder lumen WITTENBERG (1958) also presented evidence to show that bubbles take part in gas deposition It has been shown that a high CO_2 content enhances the initiation of cavitation (BLINKS TWITTY and WHITAKER 1951) The high CO_2 concentration during secretion may therefore have importance to facilitate the formation of bubbles thus increasing the diffusivity of the blood to bladder barrier

Gas secretion in deep sea fishes SCHOLANDER (1954) argued that the effects of an acid added to the venous *rete* blood could produce O_2 by counter current multiplication only at pressures at which the lowered pH could dissociate oxyhemoglobin For this reason the above mentioned system has been held inadequate to explain O_2 secretion in many deep sea fishes where the pO in the bladder is too high for pH to influence the hemoglobin bound O_2 It seems appropriate to reconsider this problem since it constitutes the major challenge to acceptance of the acid induced counter current multiplication theory as the general mechanism of gas secretion As pointed out by KOCH (1934) an acid will influence not only the dissociation of hemoglobin but it will also have a salting out effect on gases in general. The quantitative consequences of this have been pointed out by KUHN and KUHN (1961) Take as an example a fish in which a lowered pH will dissociate oxyhemoglobin at pressures only up to 50 atm In this case the pH effect will bring the O_2 pressure in the bladder up to 50 atm in the first few millimeters of the *rete* from the heart pole while the rest of the *rete* will continue the O_2 concentration but now based on the salting out effect KUHN and KUHN (1961) found that the p_{O_2} in the bladder of such a fish could reach 1 500 atm They also established that the rate of O_2 secretion

— assuming a constant blood flow — is determined by the difference in O_2 content between venous and arterial blood at the heart pole of the *rete*. The rate of O_2 secretion *e g* at 50 atm will be the same therefore whether the pH effect extends to 50 or 100 atm as long as the effect is the same below 1 atm. This is evident from the fact that the salting out effect is proportional to the dissolved gas. At 50 atm blood will contain roughly 150 vol % O_2. A 1 % reduction in solubility would split off 1 6 vol % while at 1 atm it splits off only 0 03 vol %. We must therefore conclude that gas secretion also in deep sea fishes is satisfactorily explained by a counter current multiplication of a primary gradient induced by the addition of lactic acid to the venous branch of the *rete*, and that this also enables the gas gland to secrete O against pressures which nullify the effect of low pH on hemoglobin bound O_2.

Terminology. The activity of the gas gland is commonly referred to as a secretion. According to modern criteria this term implies an energy requiring cellular synthesis and concentration of the gas to be deposited. Some authors also use the term active transport which would imply that the gas is transported across some cell membrane or layer against its concentration gradient. None of these criteria seem to be fulfilled however, if gas is concentrated by an acid induced counter current multiplication. The term gas secretion has nevertheless been maintained in this paper, since no adequate substitute has been found.

During these investigations I have benefited from Prof R FANGE's experience in swim bladder physiology. I much appreciate expert assistance from Mr O IVERSEN with the experimental equipment, from Miss I GJOEN and Mr O BRYNHILDSRUD with the illustrations and from Miss E BRADSTREET and Miss R. MORK with the lactic acid analyses. Special thanks are due to Prof P F SCHOLANDER and Dr A GRENNELL for their criticism and suggestions during the preparation of the manuscript. Mrs A. MOE has shown admirable patience in typing the manuscript several times. The investigations have been supported by A/S Norsk Varekrigsforsikrings Fond.

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Acta physiol scand 1963 59 242—254

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An Improved Method for Fluorimetric Determination of Small Amounts of Adrenaline and Noradrenaline in Plasma and Tissues

By

JAN HAGGENDAL

Received 22 February 1963

Abstract

HAGGENDAL, J *An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues* Acta physiol scand 1963 59 242—254 — By the introduction of several modifications of the trihydroxyindole method for the determination of adrenaline and noradrenaline appreciable improvement of the sensitivity has been obtained The blank values have been considerably reduced and stabilized by substituting dimercaptopropanol (BAL) in sodium sulfite solution for ascorbic acid The eluate volume has been reduced and the degree of purification has been increased by a modified ion exchange procedure (*Dowex 50 W X8*) Deproteinization of plasma before the column procedure could be omitted When this procedure was applied to 10 ml plasma obtained from normal p[illegible]ns at rest noradrenaline spectra with two activa[illegible]ually obtained The average concentration of the [illegible] was 0 3 ± 0 11 μg per litre of plasma Normally no ad[illegible] found Noradrenaline occurred in plasma in both free and [illegible]d form

The chemical determination [illegible]f the small amounts of adrenaline (*A*) and noradrenaline (*NA*) in plasma seems at *present only to be possible by fluorimetric* methods These methods are based on one of two reactions the trihydroxyindole (THI) reaction (EHRLÉN 1948 LUND 1949 a b c) and the ethylenediamine condensation (ED) reaction (NATELSON LUGOVOY and PINCUS 1949 WEIL MALHERBE and BONE 1952) However, the ED reaction appears to be less specific than the THI reaction (EULER 1956 VALK and PRICE 1956 and others)

In the THI methods A and NA are oxidized to adrenochrome and noradrenochrome which in alkali are rearranged to strongly fluorescent trihydroxy indoles adrenolutine and noradrenolutine, respectively which are estimated fluorimetrically The fluorescence of the lutines is stabilized by the addition of ascorbic acid Several methods for determination in plasma have been developed *e g* LUND (1949 a b c) PRICE and PRICE (1957) COHEN and GOLDENBERG (1957 a b) VENDSALU (1960) The methods differ in many details For review see EULER (1962)

VENDSALU (1960) has shown A and NA values in plasma samples from different parts of the circulation during rest during muscular work and after some drugs The method was with some modifications based on the method for A and NA determination in tissues by BERTLER CARLSSON and ROSENGREN (1958) The oxidation of A and NA to chrome derivatives was performed with potassium ferricyanide at pH 6 5 For the differentiation between A and NA the difference in the activating spectra of the fluorophores were made use of

In preliminary experiments based on the method of VENDSALU (1960) certain limitations of the determination technique were observed

The amount of A or NA is calculated from the difference between the sample and the tissue blank Since the reading of the sample is but slightly higher than that of the tissue blank variations of both values affect the result obtained Different types of tissue blanks also gave different values One type the non oxidized tissue blank in which the A and NA had been protected from oxidation by adding the antioxidant at an early stage (BERTLER *et al* 1958) showed higher values than the faded tissue blank in which the oxidation had gone so far that the fluorescent compounds had been destroyed

Fluorescent material from the cation exchange resin was found to interfere and was partly responsible for the difference between the blanks However the most important factor responsible for the high blanks was found to be the ascorbic acid

Consequently we have tried 1) to increase the fluorescence intensity of the A and NA in the sample 2) to eliminate the fluorescent material derived from the resin and 3) to find a more suitable antioxidant than ascorbic acid

Apparatus and reagents

Aminco-Bowman spectrophotofluorometer (all wa elengths given in this paper are uncorrected instrumental values) All the spectra were recorded on a Moseley Autograf For the ion-exchange procedure the apparatus described by BERTLER *et al* (1958) was used.

Ascorbic acid from several sources has been used in this laboratory both before and after recrystallization without any marked differences in the results Dimercaptopropanol (BAL) was obtained from Boots Pure Drug Company Nottingham England

For the column chromatography Dowex 50 W X8 was used after the finest and coarsest particles of the resin had been removed and the resin rinsed several times with

3 N HCl and 2 N NaOH containing 1 per cent disodium ethylenediamine tetra acetate (EDTA) Commercially available reagent grade chemicals were used The water was glass distilled The HCl was distill d three times which seemed to improve the re coveries

I Assay of A and NA

The pH of the eluate as a rule 2 3 ml N HCl, was adjusted to 0 5 with 5 N K_2CO_3 (*indicator paper*) after 0 60 ml of the eluate had been *removed* for use in the non oxidized tissue blank (see below)

The sample To 0 70 ml of the neutralized eluate 0 10 ml 0 1 M phosphate buffer, pH 6 5 was added After 0 02 ml 0 0025 *per cent* CuCl $2H_2O$ solution had been added the oxidation was performed by addition of 0 05 ml 0 25 per cent $K_3Fe(CN)_6$ After 3 min the oxidation was stopped with 0 125 ml of a solution of 0 5 per cent BAL in a sodium sulfite solution *containing* 5 g Na_2SO_3 7 H_2O in 10 ml water This was immediately followed by the addition of 0 2 ml 10 N NaOH

The standard A and NA 0 010 ml of a solution of 1 0 μg catechol amine base per ml in 0 0005 N HCl was added to 0 7 ml water and *treated as a sample*

The internal standard (IS) 0 010 μg A or NA was added to a sample which was treated as above

The non oxidized tissue blank 0 60 ml of the eluate was added to a freshly prepared mixture of 0 1 ml of the phosphate buffer and 0 125 ml of the BAL-Na_2SO_3 solution $CuCl_2$ $K_3Fe(CN)_6$ and NaOH were added as above and they were followed by 0 10 ml 5 N K_2CO_3

The faded tissue blank The blank was treated as a sample After 3 min of oxidation *however, only the* 10 N NaOH was *added After another 10 min* the BAL Na_2SO_3 solution was added

The sample and the blanks were read after about 10 min in an Aminco Bowman spectrophotofluorometer at 400/515 and 450/515 mμ (activating/ fluorescent wavelengths) As an alternative to the latter wavelength combina tion they may be read at 330/515 mμ or 400/550 mμ The spectra were recorded and the amounts of A and NA were calculated (see below)

The procedure is outlined in Table I An example of a determination on plasma of a healthy person at rest is given

Comments

The sample volume The total volume was kept as low as 1 2 ml to reduce the diluting effect on the fluorescence of the reagents The standard cuvettes of the spectrophotofluorometer could still be used

The oxidation The spontaneous oxidation of A and NA in an alkaline solu tion to fluorescent compounds (LOEW 1918, PAGET 1930 GADDUM and SCHILD 1934) has long been utilized for fluorimetric determination The fluorescence is unstable but can be stabilized with ascorbic acid (EHRLÉN 1948 LUND

Table I Example of analysis of A and NA in an eluate from a blood sample drawn from the brachial artery of a healthy man in rest (The spectra are shown in Fig 2)

Reagents and solutions in ml	Standard NA	Standard A	RB	Sample	IS	Nonoxidized TB	Faded TB
H_2O	0.70	0.70	0.70	—	—	—	—
Phosphate buffer	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Eluate neutralized	—	—	—	0.70	0.70	0.60	0.70
NA	0.010	—	—	—	*0.010	—	—
A	—	0.010	—	—	0.010	—	—
$CuCl_2$	0.02	0.02	0.02	0.02	0.02	0.02	0.02
I $K_3Fe(CN)_6$	0.05	0.05	0.05	0.05	0.05	0.05	0.05
II BAL-Na_2SO_3 added 5 min after I	0.125	0.125	0.125	0.125	0.125	*(0.125)	—
III NaOH 10 N added 10 sec after II	0.20	0.20	0.20	0.20	0.20	0.20	0.20
IV BAL-Na_2SO_3 added 10 min after III	—	—	—	—	—	—	0.175
V K_2CO_3 5 N added after III	—	—	—	—	—	0.10	—
Fluorescence intensity at							
400/515 mμ	20	17	0.6	1.8	22	1.2	1.0
450/515 mμ	2.6	10	1.0	1.5	3.8	1.4	1.5

Alternatingly In the example NA was used

0.60 ml of the non neutralized eluate.

The BAL-Na_2SO_3 solution was added before the eluate

Other wavelengths may also be used see text

The NA standard was set to 20 fluorescence units.

The A standard was set to 10 fluorescence units.

Calculation The A (= $x \cdot 10$ ng) and NA (= $y \cdot 10$ ng) in Sample were calculated by solving the equations

$$(17-0.6)\,x + (20-0.6)\,y = 1.8 - \frac{(1.2+1.0)}{2} \quad \text{(Readings at 400/515 m}\mu\text{)}$$

$$(10-1.0)\,x + (2.6-1.0)\,y = 1.5 - \frac{1.4+1.5}{2} \quad \text{(Readings at 450/515 m}\mu\text{)}$$

$$x = 0.00$$
$$y = 0.04$$

1949) But higher intensity is obtained when the oxidation is performed at a more neutral pH with added oxidants

Fluorescence with good intensity stability and reproducibility was however obtained by alkaline treatment in a two-step procedure of A and NA in pure solutions At first a weak alkaline solution e g 0.1 M ammonium hydroxide or borate buffer, pH 8.5 was added Some cupric chloride (10^{-4} g/ml) was also present Five minutes later EDTA and ascorbic acid were added Then a

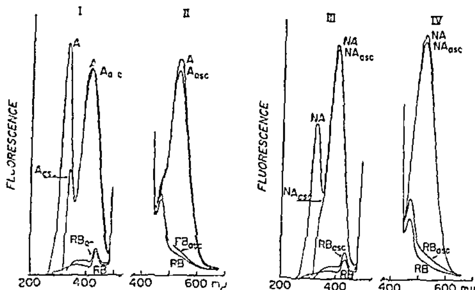


Fig 1 Activation and fluorescence spectra of 10 ng adrenaline and 10 ng noradrenaline (after oxidation and rearrangement in alkali) before and after the addition of ascorbic acid I Activation spectrum Fluorescence wavelength 525 mμ II Fluorescence spectrum Activating wavelength 420 mμ III Activation spectrum Fluorescence wavelength 515 mμ IV Fluorescence spectrum Activating wavelength 400 mμ
A_{asc}, NA_{asc}, RB_{asc} the spectra of adrenaline (A) noradrenaline (NA) and reagent blank (RB) after the addition of ascorbic acid ($2 \cdot 10^{-4}$ g/ml)

strong alkaline solution *e g* 5 N NaOH followed The reproducibility was not as good at the oxidation of tissue extracts as in pure solutions so at present the oxidation is performed with potassium ferricyanide at a pH of 6 5

In earlier methods the oxidation is often performed in the presence of added small amounts of zinc sulfate The effect of zinc sulfate on the oxidation of pure solutions of A and NA at pH 6 5 was compared with the effect of cupric chloride and with the effect when no metals were added No effect of the zinc sulfate was found The zinc sulfate addition may however be of value in stabilizing the oxidation of tissue extracts

The addition of Cu^{++} ions appears to interfere with the assay in different ways First, the oxidation of A and NA to chrome derivatives appears to be accelerated Using Cu^{++} concentrations up to 10^{-5} g/ml these derivatives appeared to be relatively stable at neutral or weakly alkaline pH Second Cu^{++} ions have been found to cause weakening of the fluorescence of the lutines in strong alkali This effect is partly reversible If the fluorescence of lutines in alkali was brought to disappear by the addition of Cu^{++} ions it could then be brought to partly reappear by the addition of EDTA within a few minutes This initial reversible weakening of the fluorescence is possibly due to formation of complexes between Cu^{++} ions and the lutines However the protective action of EDTA was no longer detectable after a more prolonged

exposition of the lutines to Cu^{++} ions indicating irreversible conversion of the lutines to non fluorescent compounds

If EDTA was added in sufficient concentrations before the Cu^{++} ions complete protection was obtained This was also true for the BAL-sodium sulfite solution used in the present method It is interesting to note that ascorbic acid when added in the usual high concentration did not protect the lutines from the effect of Cu^{++} ions

In the present procedure a small amount of cupric chloride has been added as an attempt to keep the effect of metal ions under more constant control In fact addition of Cu^{++} ions seems to increase the reproducibility of the results

The stabilization of the lutines Although the use of ascorbic acid for the stabilization of the fluorescence is well established it has definite disadvantages Above all the use of abscorbic acid gives rise to high and variable blank values The intensity of the blank values increase with increasing content of ascorbic acid According to EULER and LISHAJKO (1961) the addition of ethylenediamine to the ascorbic acid improves the stability of the sample and the blanks

Within certain wavelength ranges the use of ascorbic acid will give light absorbance (Fig 1)

Instead of ascorbic acid other agents were tried for the stabilization The best results were obtained with the following compounds all of them containing sulphur hydrogen sulfide sodium sulfide BAL sodium pyrosulfite and cysteine hydrochloride

The most reproducible results were obtained with a freshly prepared (not more than 2 hours old) solution of BAL 0 5 per cent in a sodium sulfite solution 5 g Na_2SO_3 7 H_2O ad 10 ml water (The BAL was cautiously mixed with the Na_2SO_3 solution until a clear solution was obtained The sodium sulfite solution should be less than 6 days old All solutions containing BAL were handled in a hood)

The fluorescence obtained was as high as with ascorbic acid The values of the blanks were smaller and more stable The reproducibility was good

The activating spectra of both A and NA had two peaks one at 420/525 mμ for A and at 395/515 mμ for NA (activating/fluorescent wavelengths) and another at 330/525 mμ for A and at 325—330/515 mμ for NA When ascorbic acid was added in the concentration generally used ($2 \cdot 10^{-4}$ g/ml) the activating peaks at 330 mμ almost completely disappeared A small rest was seen for A (Fig 1)

The presence of two activating peaks has proved to be of value in question of identity particularly when the intensity of the fluorescence was weak The ratio between the two peaks were different for A and NA This could be utilized for differentiation between A and NA

The blanks As mentioned above the blanks were lower and more stable when the BAL-sodium sulfite solution was used However, there still was a

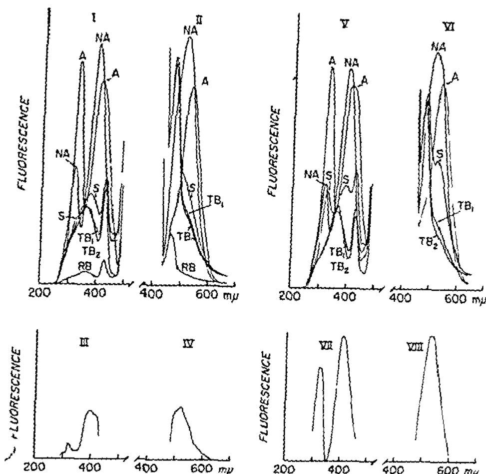


Fig 2 Activation and fluorescence spectra of plasma samples (after oxidation and rearrangement in alkali) obtained from the brachial artery of a healthy man in rest before and after insulin I and V Activation spectra Fluorescence wavelength 515 mμ II and VI Fluorescence spectra *Activating wavelength 400 mμ I and II Spectra from the sample obtained during* rest. V and VI Spectra from the sample obtained 30 min after insulin 0 1 I U per kg body weight i v The volumes of the plasma samples 10 and 13 ml respectively S plasma sample TB_1 non oxidized tissue blank TB faded tissue blank A adrenaline standard 10 ng NA noradrenaline standard 10 ng RB reagent blank.

At the recording the sensitivity was about 4 times higher for the samples and tissue blanks than for the standards When the standard NA at 400/515 mμ was set to 20 fluorescence units the fluorescence intensities were standard A 17 and the RB 0 6 the sample during rest 1 8 with TB 1 2 and TB 1 0 and the sample after insulin 3 4 with TB 1 3 and TB 1 0 fluorescence units

The difference of the intensity between the samples and the mean values of the corresponding two types of tissue blanks were obtained from the figures These values after having been doubled are shown under the corresponding figures Figures III and IV show activation and fluorescence spectra typical for NA Figures VII and VIII show an increase of the fluorescence intensity The change in ratio between the two activating peaks indicates that the increase is due to A

Calculated values (see Table I for calculation) During rest 0 3 μg NA per litre plasma No A was found After insulin 0 3 μg NA and 0 5 μg A per litre plasma

difference between the non oxidized tissue blank and the faded tissue blank the former being higher This seemed to be partly due to spontaneous oxidation during the adjustment of the pH of the eluate When the blanks were prepared in the way now used this risk was eliminated The faded tissue blank tended to be too low since more of the fluorescent compound derived from the strong cation exchange resin was destroyed in the blank than in the sample (HAGGENDAL 1962) This interference could be reduced by washing the columns intensely and omitting the first portion of the eluate The spectra of the sample and the blanks were recorded for control of e g the spontaneous oxidation in the non oxidized tissue blank For the calculation the mean values of the two blanks were used

It is essential that the samples are well separated from solutions containing BAL Otherwise inhibition of the oxidation may be observed Thus the non oxidized tissue blank to which the BAL-sodium sulfite solution is added at an early stage should preferably be kept in a hood well separated from the samples

The internal standard In general there was good agreement between the usual standard and the internal standard but occasionally deviations were observed Such deviations should be referable to the composition of the eluate with respect to pH ionic strength and/or presence of interfering material derived from the resin or the tissue extract In order to keep these sources of error under control internal standards were used routinely As a rule the determinations were discarded if the difference between the internal standard and the sample was less than 80 per cent or more than 120 per cent of the difference between the pure standard and the reagent blank

The calculation of A and NA

For the calculation of the A and NA amounts in the plasma the differences of the activating spectra of the catechol amines were utilized by BERTLER *et al* (1958) and VENDSALU (1960) Readings were thus performed at the activating wavelengths 400 and 450 mμ at a fluorescence wavelength of 515 mμ In the present experiments however when the fluorescence intensity of the sample was very low the activating spectrum showed a peak at about 400 mμ (Fig 2) This peak was also found in the blanks and even in pure water and was related to the light scatter As light scatter is variable there may be disturbances in the readings at 450 mμ when very low amounts of catechol amines are present

As another possibility the different fluorescence spectra of A and NA may be utilized for the differentiation

As a third possibility the ratio between the two activating peaks obtained with the BAL sodium sulfite solution may be used for the calculation In this case it is particularly essential that internal standards for both A and NA are used since the intensity of the activating peaks at 330 mμ appeared to be

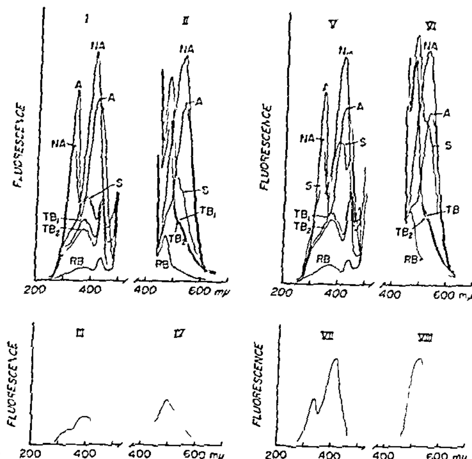


Fig 3 Activation and fluorescence spectra of plasma samples (after oxidation and rearrangement in alkali) obtained from the antecubital vein of a healthy man at rest and during muscular work. I and V Activation spectra Fluorescence wavelength 51ɔ mμ II and VI Fluorescence spectra Activating wavelength 400 mμ I and II Spectra from the sample 15 ml plasma obtained during rest V and VI Spectra from the sample 12 ml plasma obtained during muscular work, bicycle ergometer [illegible]00 kpm for 15 min.

For abbreviations and further explanation see Fig 2

When the intensity of standard NA at 400/ɔ15 mμ was set to 20 fluorescence units the following values were obtained the sample during rest 2 0 with TB_1 1 4ɔ and TB_2 1 05 the sample during work 3 3 with TB_1 1 3 and TB_2 1 1ɔ

After having been doubled the values of the differences between the samples and the mean values of the corresponding two types of tissue blanks also shown under the corresponding figures

Calculated values During rest 0 2 μg NA per litre plasma During work 0 6 μg NA per litre plasma No measurable amounts of A were found

more easily reduced by interfering compounds than the peaks at about 400 mμ This interference is probably due to light absorption

As spectra are recorded routinely, the importance of these various disturbing factors can be evaluated thus facilitating the choice between the three alternatives A figure showing the difference of the fluorescence intensity between

Table II Preparation of the plasma extract

1 18 ml blood was collected in an ice cold plastic centrifuge tube (International Equipment Company original tube) containing 2 ml 1 per cent EDTA in 0 9 per cent NaCl solution
2 Within 1 hour the sample was centrifuged at about 10 000 × *g* for 20 min at + 4 C
3 The plasma was sucked off and the volume measured.
4 The bulk of proteins was precipitated with 0 1 volume of 4 N perchloric acid.
5 The sample was centrifuged at about 10 000 × *g* for 15 min after it had been allowed to stand for about 30 min.
6 The volume of the supernatant was measured

The plasma extract was now ready to be passed through a column after adjustment of the pH The acid supernatant could however now also be left in a freezing box for several weeks without any measurable destruction of the catechol amines

the sample and the tissue blanks at *e g* every 10th mμ is here often of value (See Figures 2 and 3) In many cases differentiation will then be found unnecessary as the plasma samples will often be found to contain almost exclusively noradrenaline In such cases readings at 400/515 mμ will be sufficient Adrenaline has been found to occur in measurable quantities only in adrenal venous blood or under special conditions such as insulin hypoglycemia

II The Column Procedure

For the purification and concentration of A and NA essentially the method of BERTLER *et al* (1958) has been used in the way VENDSALU (1960) has applied it for determination in plasma For example 1 per cent EDTA in physiological saline has been used as the anticoagulant Perchloric acid (0 4 N) has been used for precipitating the bulk of proteins and strong cation exchange resin columns for the purification and concentration of A and NA Elution has been performed with N hydrochloric acid

The following modifications of the column procedure have been introduced a) the dimensions of the columns have been modified b) the resin has been Dowex 50 W X8 in Na^+ form c) less acid has been used for the elution, and d) the first portion of the eluate has been omitted

In Table II the treatment of the plasma up to the column procedure is given In Table III the column procedure is described

A simplified way of preparing the plasma extract was also recently tried The blood was collected and centrifuged as above The plasma was then without deproteinization passed through the column After rinsing according to Table III but with 20 ml more water containing 0 1 per cent EDTA the elution was performed with the acid The procedure was easy and faster than the one in Table II The results of the two procedures were in good agreement

After acid hydrolysis of deproteinized plasma (at 100 C in N perchloric acid for 10 min) the values of A and NA increased In plasma as in urine A

Table III The column procedure

Columns of Dowex 50 W X8 diameter 2 7 mm length 35 mm were used in Na^+ form. Before the plasma extract was passed through the column was pretreated with

1 15 ml 2 N NaOH containing 1 per cent EDTA
2 About 40 ml water to remove the NaOH
3 20 ml 2 N HCl
4 About 50 ml water to remove the acid.
5 15 ml 0 1 M phosphate buffer pH 6 5 containing 0 1 per cent EDTA
6 5 ml water containing 0 1 per cent EDTA

The pH of the plasma extract was adjusted to about 5 5 with 5 N K_2CO_3 After centrifugation 10 000 × g for 10 min at +4 C

1 The plasma extract was passed through and the column washed with
2 20 ml water containing 0 1 per cent EDTA.
3 15 ml 0 1 M phosphate buffer pH 6 5 containing 0 1 per cent EDTA.
4 10 ml water
5 The elution was performed with N HCl The eluate was omitted until the pH was less than 2 5 The following 2 3 ml contained the A and the NA

The rates of the solutions for the washing were 15—20 ml per hour The rates for the plasma extract and for the elution were about 10 ml per hour

The column could be used for many determinations if it first was washed with water and then as described above

id NA thus seem to occur both in free and in conjugated form The bound appeared to be at least as large as the free form

By a two column procedure the free and the bound fractions can be determined separately The plasma sample was first passed through a column as described above The free catechol amines were adsorbed and could be estimated after elution The plasma which had passed through together with 15 ml water containing 0 1 per cent EDTA for rinsing were hydrolysed in acid and passed through a new column after adjustment of the pH After rinsing the bound catechol amine fraction which had been liberated by hydrolysis, could be eluted and estimated Results of further studies of free and bound A and NA in plasma will be published elsewhere

Recovery and Reproducibility

Since the A and NA levels in plasma generally are very low the recovery of added small amounts of A and NA has been of particular interest After addition of 50 ng A and 50 ng NA to 10 ml normal plasma the determination was performed with the column procedure and the analysis of the eluate as described above Ten such determinations were performed The mean recovery of the sum of the added A and NA was 71 per cent with a standard deviation

of 16 per cent The amount of recovered A was about the same as that of NA Nine determinations were performed with 10 ng A and 10 ng NA added The mean recovery was 73 per cent with a standard deviation of 29 per cent

From a plasma sample twelve 10 ml fractions were treated separately according to the method described above One sample was omitted since at the oxidation the difference between the internal standard and the sample was less than 80 per cent of the difference between the pure standard and the reagent blank In the 11 samples no A was found The mean concentration of NA was 0 23 ± 0 075 μg per litre plasma which means a standard deviation of 33 per cent

Sensitivity

With this method typical activating and fluorescent peaks of 0 5—0 1 ng A and NA in pure solutions may be obtained

It should be possible to increase the sensitivity by further reducing the volumes at the oxidation procedure and by reading in microcuvettes in the spectrophotofluorometer In that case the column procedure must also be changed so it can accommodate the reduced volumes

With the present method as a rule spectra of NA were found in the assay of 10 ml plasma from a person in rest (see Fig 2) Less plasma *e g* 5 ml could be used when the NA level was higher *e g* during muscular work

Specificity The increased sensitivity of the method the possibility of utilizing two peaks in the activatin spectra of A and NA and the reduction and stabilization of the blank values should together mean an increase of the specificity

By lengthening the column the chromatographic separation (Section II) can be made more specific Even complete separation between A NA and dopamine and their 3 O methylated derivatives could be obtained with columns of this type (HAGGENDAL 1962) But the duration of the procedure will also increase The columns used at present appear sufficient for the purification and the concentration of the plasma catechol amines

With this method some results of VENDSALU (1960) have been confirmed The NA concentration in plasma was thus found to increase during muscular work (Fig 3) Details of these experiments will be published elsewhere When the hypoglycemia after insulin was most marked the A level in plasma was the highest (Fig 2) The lowest values of A or NA were found in samples from the hepatic vein The mean NA concentration in plasma from the antecubital vein or the brachial artery of 13 persons in rest was 0 3±0 11 μg per litre (the values corrected for the recovery) No measurable amounts of A were found

The method may also be used for other tissues extracts and body fluids than plasma It has for instance been used for determination of small amounts of A and NA in small pieces of brain tissue

The work was supported by the Medical Faculty of Göteborg and by the Directorate of Life Sciences AFOSR Office of Aerospace Research United States Air Force monitored by the European Office Office of Aerospace Research under Grant No AF EOAR-61-41

For technical assistance I am indebted to Mrs INGA LILL HEDBERG and Mrs BIRGITTA SANDLIN

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Acta physiol scand 1963 59 255—260

From the Department of Pharmacology University of Goteborg Sweden

The Presence of Conjugated Adrenaline and Noradrenaline in Human Blood Plasma

By

JAN HAGGENDAL

Received 22 February 1963

Abstract

HAGGENDAL J *The presence of conjugated adrenaline and noradrenaline in human blood plasma* Acta physiol scand 1963 *59* 255—260 — The presence of conjugated adrenaline and noradrenaline in blood plasma is shown As a rule 2 to 3 times more conjugated than free noradrenaline was found When the free noradrenaline and adrenaline plasma levels were increased during muscular work and during insulin hypoglycemia respectively the levels of conjugated catecholamines did not markedly change After the oral administration of 5 mg adrenaline to a healthy man high amounts of conjugated adrenaline was found in the plasma and in the urine

In the urine of man and several other mammalian species catecholamines are present in both free and conjugated form On the basis of the results of RICHTER (1940) and RICHTER and MACINTOSH (1941) conjugation was thought to be an important pathway for the metabolism of the catecholamines When in these experiments a large dose of a catecholamine was ingested most of the compound was excreted in the urine as conjugates The conjugates are suggested to be etheral sulphates or glucuronides When small doses of adrenaline (A) or noradrenaline (NA) were parenterally administered however only small amounts were excreted in conjugated form (EULER and LUFT 1951 EULER LUFT and SUNDIN 1953 SCHAYER 1951) Most of the given doses were metabolized in other ways chiefly by O methylation and deamination For review see AXELROD (1959)

The conjugation is generally thought to occur in the liver but other possibilities have been mentioned (RICHTER and MACINTOSH 1941) The presence of A or NA in conjugated form in plasma seems not to have been shown

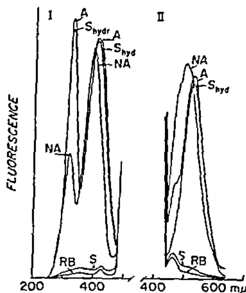


Fig 2 Activation and fluorescence spectra of plasma samples (after oxidation and rearrangement in alkali) from the ant cubital vein 60 min after the ingestion of 5 mg adrenaline I Activation spectra, Fluorescence wavelength 515 mμ II Fluorescence spectra. Activating wavelength 400 mμ A adrenaline standard 10 ng NA noradrenaline 10 ng RB reagent blank S a non hydrolyzed plasma sample 14 5 ml, (showing the free catecholamines) S hydr a plasma sample 12 ml hydrolyzed for 10 min (showing the sum of free and conjugated catecholamines)

The samples were recorded at about 4 times *lower* sensitivity than the standards

Calculated values μg per litre plasma

The non hydrolyzed sample	NA	0 5
	A	0 0
The hydrolyzed sample	NA	0
	A	3 0

VENDSALU (personal communication) using some modification of his method (VENDSALU 1960) has also found increased amounts of catecholamines in plasma after acid hydrolysis

The levels of conjugated catecholamines were in some experiments estimated under conditions where the amounts of free catecholamines were increased The increase was obtained for the free plasma NA during muscular work and for the free A during insulin hypoglycemia

When two healthy men performed muscular work (bicycle ergometer 600 and 900 kpm) for 30 min their plasma levels of free NA increased 4 and 6 times, respectively compared with the levels at rest Their levels of conjugated NA were only slightly changed They increased less than 50 per cent

That the plasma level of A is increased after insulin hypoglycemia is well known (see VENDSALU 1960) In Table I the results are shown of the determination of free and conjugated A and NA in plasma samples from the brachial artery and hepatic vein before and 30 min after the intravenous administration of 8 I U insulin (0 1 I U per kg body weight) to a healthy man In the blood from *the brachial artery* the level of conjugated NA was twice as high as the level of free NA. After insulin free A was found in addition to NA The level of conjugated catecholamines did not increase after the insulin but there seemed also to be traces of conjugated A In the blood from *the hepatic vein* the levels of free catecholamines were very low even after insulin This is in agreement with the results of VENDSALU (1960) Taking samples from different parts of the circulation in the human body he found the lowest values in the hepatic vein From the results shown in Table I it seems that the liver com

pletely metabolizes the free catecholamines even when they have increased after insulin When the plasma from the hepatic vein was hydrolyzed, conjugated NA was found After insulin there was no increase of the level of conjugated catecholamines but perhaps a slight addition of A to the NA The levels of conjugated catecholamines were not higher in the hepatic vein than in the artery

Two more experiments of this type have been performed one on a healthy man and the other on a diabetic The results were in agreement with those in Table I

When the free NA increased during muscular work and the free A increased during insulin hypoglycemia there was no marked increase of the conjugated catecholamines

Since RICHTER (1940) and RICHTER and MACINTOSH (1941) obtained high amounts of conjugated A in the urine after oral administration of A the levels of free and conjugated A in plasma were estimated after ingestion of 5 mg A. No evident effects on pulse rate or blood pressure were obtained Every 20 min blood samples were drawn from the antecubital vein and assayed for free and conjugated A and NA. No free A was found in the samples but extremely high values were obtained for conjugated A The sample after 60 min which was the one with the highest intensity contained 30 μg A per litre plasma (Fig 2 shows the spectra of the free and the conjugated catecholamines in this sample) Increased amounts of conjugated A were also found in the urine Within two hours more than half of the administered dose of A was found as conjugated A in the urine

The most important pathway for the inactivation of the circulating A and NA is supposed to be O methylation in the liver (AXELROD 1959) In the present experiments the role played by conjugation appeared to be small when the free A and NA levels were increased by insulin and muscular work respectively After the oral administration of A however conjugation was the predominant metabolic pathway Only a small fraction of the administered dose was found as 3-O methylated A. Since the ability of the liver to O methylate is supposed to be large it is possible that the orally administered A was conjugated before it reached the liver The A may have been conjugated by the intestinal flora or in the intestinal mucosa

For placing the human material at my disposal and carrying through the catheterisations I am greatly indebted to A. CARLSTEN M D Professor of Clinical Physiology A. SVANBORG M D Associate Professor of Medicine and L. WERKO M D Professor of Medicine University of Goteborg Sweden. I am also most indebted to Dr E. H. GOENDAL for his valuable help

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Acta physiol. scand 1963 59 261—268

From the Department of Pharmacology University of Goteborg Sweden

The Presence of 3-O-Methylated Noradrenaline (Normetanephrine) in Normal Brain Tissue

By

Jan Haggendal

Received 22 February 1963

Abstract

Haggendal, J *The presence of 3-O methylated noradrenaline (normetanephrine) in normal brain tissue* Acta physiol scand 1963 *59* 261—268 — In the brain of normal pigs and rabbits normetanephrine was found in amounts corresponding to about 15 per cent of the noradrenaline values After reserpine and after a catechol O methyl transferase inhibitor the levels of normetanephrine and of methoxytyramine were strongly reduced while initially the levels of noradrenaline and dopamine were normal In extracts from the hypothalamus of man normetanephrine was also found O methylation seems to be an important pathway in the normal metabolism of noradrenaline in brain

In the past decades several metabolic pathways for the catecholamines have been demonstrated *e g* oxidative deamination by monoamine oxidase (MAO) conjugation and 3 O methylation by O methyltransferase (COMT for review see Axelrod 1959) The relative importance of the different inactivating enzyme systems are still under discussion The role of COMT appears to be important for the metabolism of the circulating adrenaline (A) and noradrenaline (NA) (Axelrod *et al* 1958 Axelrod 1960)

In the central nervous system both MAO and COMT are present but deamination has been suggested to be the principal pathway for the metabolism of NA The brain NA level has been found to increase after administration of MAO inhibitors but not after the COMT inhibitor pyrogallol (Crout Creveling and Udenfriend 1961) The presence of 3 O methylated NA (normetanephrine NM) in normal brain tissue has not been demonstrated

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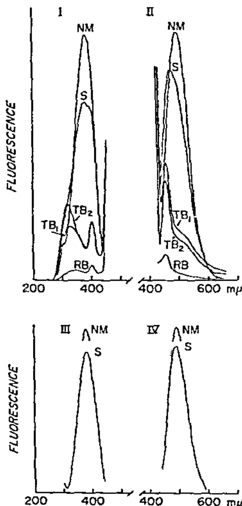


Fig 2 Activation and fluorescence spectra of the eluate fractions nos 17 to 21 marked in Fig 1 after oxidation and rearrangement in alkali.

I Activation spectrum. Fluorescent wavelength 485 mμ

II Fluorescence spectrum Activating wavelength 390 mμ

NM normetanephrine standard. RB reagent blank.

S the sample TB1 non-oxidized tissue blank.

TB faded tissue blank.

NM* the top of the NM standard from which the RB is subtracted

S the sample from which the mean values of the TB1 and TB2 are subtracted.

The sample was recorded at about 4 times higher sensitivity than the standard

normal brain both enzyme (COMT) and substrate (NA) are available 3 O methylation normally occurs in the brain since the 3 O methylated derivative of dopamine methoxytyramine (MT) was demonstrated in the brain of pig cow cat and sheep (CARLSSON and WALDECK 1963) For these reasons attempts were made to demonstrate the presence of NM in brains of man pig and rabbit

Methods

The brain tissue was as soon as possible homogenized in 0 4 N perchloric acid according to BERTLER CARLSSON and ROSENGREN (1958) After centrifugation at about 10 000 *x g* at 4 C for 10 min the pH of the extract was adjusted to 5 5 with 5 N K_2CO_3 The solution was recentrifuged at 4 C It was then passed through a column of strong

Table II *Normetanephrine (NM) noradrenaline (NA) dopamine (DA) and methoxytyramine (MT) in brain* (μg per gram brain tissue The NM, NA and DA values corrected for the recovery 85 82 and 61 per cent, respectively)

	NM	NA	DA	MT
Man. Hypothalamus				
38-years-old man 48 hours after death. Heart failure	0 12	0 83	0 75	0 30
73-years-old man, 40 hours after death. Myocardial infarct	0 04	0 16	traces	traces
79 years-old woman, 16 hours after death. Cancer of uterus. The intermediate part of the hypothalamus was assayed.	0 24	1 62	0 00	0 00
67 years-old man 9 hours after death. Cancer of stomach	traces	0 32	0 46	0 00
Pig				
Hypothalamus (4 animals)	0 18	1 42	0 59	—
Surroundings of hypothalamus	0 05	0 81	0 26	—
Rabbit (One rabbit per determination)				
Normal	0 044	0.20	0 25	—
	0 053	0 29	0.21	—
	0 024	0 24	0 46	0 19
	0 053	0 31	0 43	—
4 hours after a single dose of *reserpine*[2]	0 002	0 21	0.20	0 00
	0 002	0 16	0 16	—
28 hours after a single dose of *reserpine*	< 0 015	0 10	0 16	—
4 hours after the last dose of *reserpine* wich had been given daily for 7 weeks	0 005	0 01	0 00	—
	0 000	0 00	0 00	—
to 52 hours after the dose of *reserpine* which had been given daily for 7 weeks	0 001	0 002	0 013	—
	0 005	0 035	0 046	—
	0 006	0 035	0 002	—
	0 000	0 025	0 000	—
5 min after end of infusion of 2 (3 4-dihydroxyphenyl) hexanoyl amide, 500 mg per kg body weight given intravenously during 45 min	0 004	0 26	0 4[illegible]	0 00
	0 001	*0.24*	*0 39*	*0 00*

[1] — no estimation of MT was performed.

[2] The reserpine was given subcutaneously in a dose of 0.2 mg per kg body weight

cation exchange resin (Amberlite CG 120 type 2) length 10 cm diameter 3 4 mm. After rinsing with 15 ml water the elution was performed with N HCl. The eluate was collected in fractions of 1 to 2 ml. With a pressure of 0 5 m water the elution was performed at *a rate of about 4 ml per hour* In each fraction the fluorescence intensity was estimated at 285/330 mμ (activating/fluorescence wavelength uncorrected instrumental values) in an Aminco-Bowman spectrophotofluorometer In the fractions

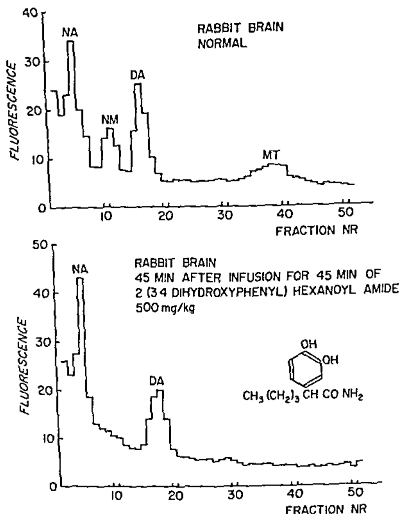


Fig 3 Column separation of the catecholamines and their 3 O methylated derivatives in brain extracts of a normal rabbit and of a rabbit pretreated with a COMT inhibitor 2 (3 4-dihydroxyphenyl) hexanoyl amide
See Fig 1 for abbreviations and description of the columns

which were of interest the more intense and specific fluorescence was estimated according to the principles of the trihydroxyindole (THI) methods For details of the column procedure see HAGGENDAL (1962)

The fluorimetric determination of NM was performed according to HAGGENDAL (1962) with some modifications Table I shows the determination procedure

NA was estimated according to BERTLER *et al* (1958) and HAGGENDAL (1963) Dopamine (DA) was estimated according to CARLSSON and WALDECK (1958) modified according to CARLSSON and LINDQVIST (1962) MT was estimated according to CARLSSON and WALDECK (1963)

Results

In Fig 1 the results of determination on an extract of hypothalamus of man are shown The fluorescence intensity in each fraction (1 1 ml) of the eluate was estimated at 285/330 mμ A complete separation of NA NM DA and MT is seen The fractions numbered 17 to 21 containing the NM were combined and the fluorimetric NM determination method used Activation and fluorescence spectra were obtained according to Fig 2 After subtraction of the blank typical NM spectra were obtained

The NM amounts obtained per gram brain tissue of man pig and rabbit are shown in Table II The NA and the DA and in some cases the MT levels are also shown In normal rabbits about 0 04 μg NM was found per gram brain tissue which is about 15 per cent of the normal NA value After reserpine the NM level was strongly reduced After a COMT inhibitor 2 (3, 4 di hydroxyphenyl) hexanoyl amide the amount of NM was reduced to less than 10 per cent of the normal value the NA level being unaffected (Fig 3) The MT level was also strongly reduced by this inhibitor while the DA level was normal

Normal rabbit brains were divided in 5 parts (hemispheres corpus striatum thalamus and hypothalamus, corpora quadrigemina with pons and medulla oblongata, cerebellum) which were assayed as described above NM and NA were found in all the 5 parts The NM concentration was not higher than 0 06 μg per gram brain tissue in any of these parts of the brain

Some determinations were performed on brain tissue extracts to which had een added 2 5 μg NM 2 5 μg NA and 5 0 μg DA The mean value of the ecovery was for NM 85 per cent For NA and DA they were 82 and 61 per cent respectively

Discussion

For the determination of the NM in the brain tissue a combination of column chromatography and spectrophotofluorometry two fairly specific methods have been used The NM from the brain tissue was found identical with synthetic NM at the column chromatography Their fluorescence maxima were also identical both the peaks at 285/330 mμ and the more specific peaks at 390/485 mμ obtained after treatment according to the THI method

The presence of NM in the normal brain tissue must mean that 3 O methylation of NA normally occurs That the amounts of NM varied considerably in the hypothalamus extracts of man may depend on several reasons such as postmortem reactions cause of death and medical treatment

The brain NM level must depend on the rate of the NM formation and the rate of the further metabolism of the formed NM The results of some drugs may thus be explained

The low NM values after the COMT inhibitor are due to inhibition of the 3 O methylation of NA The NA values obtained were normal after the COMT inhibition This does not argue against the importance of COMT in the metabolism of brain NA since a rise of the NA level obtained as a result of inhibited NA metabolism may have been prevented by an inhibiting effect of the drug on the NA synthesis

The low NM and MT values 4 hours after reserpine administration while NA and DA still remained almost normal suggest that the drug reduced the availability of NA for COMT

The high NM values after MAO inhibitors may be caused by inhibition of the deamination of the formed NM The high NA values found in these experiments may possibly be explained by product inhibition The high NA and NM values can however also be explained if deamination is an important metabolic pathway for brain NA

At present it appears to be difficult to decide if MAO or COMT plays the most important role in the metabolism of NA in the brain The presence of NM in the normal brain tissue is however strong support that O methylation of NA occurs in this tissue It is possible that one enzyme (MAO) regulates the intracellular store of NA while the other enzyme (COMT) takes part in the metabolism of the active extracellular NA.

I wish particularly to thank Miss MARGIT LINDQVIST for her kind interest in this work. For technical assistance I am indebted to Mrs. INGA LILL HEDBERG and Mrs BIRGITTA SANDLIN The work was supported by grants from the Medical Faculty of Goteborg and by the Directorate of Life Sciences AFOSR Office of Aerospace Research United States Air Force monitored by the European Office Office of Aerospace Research under Grant No AF EOAR-61 44

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Acta physiol scand 1963 59 269—283

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Non Essential Role of Prolactin in the Hormonal Restoration of Lactation in Goats with Radio Frequency Hypothalamic Lesions

By

CHARLES C GALE[1]

Received 25 February 1963

Abstract

GALE C C *Non essential role of prolactin in the hormonal restoration of lactation in goats with radio frequency hypothalamic lesions* Acta physiol scand 1963 59 269—283 — Coagulation of the median eminence by radio frequency heating in three of four lactating goats caused an abrupt onset of diabetes insipidus and a decline in milk production to 15 to 30 per cent of pre lesion levels One goat with unilateral injury in the median eminence developed diabetes insipidus briefly but showed little block of lactation In goats with depressed lactation the administration of ACTH STH T3 and insulin restored milk production to or above pre lesion levels Since no deficiency in secretion of prolactin could be demonstrated in goats with hypothalamic regulation interrupted by median eminence lesions these data are in accord with the hypothesis that prolactin secretion is controlled by a central inhibitory mechanism Diabetes insipidus developing after RF lesions was characterized by an abrupt onset of polyuria and polydipsia followed by a well-demarcated normal interphase and then by a permanent phase of elevated water turnover An amelioration of diabetes insipidus usually paralleled the decline in milk production after lesions whereas hormonal replacement particularly with ACTH and T3 restored levels of water turnover above the maximum attained acutely after lesions Thus the participation of adenohypophysial hormones is required for the full manifestation of diabetes insipidus in the goat

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In a previous study the production of proton radiolesions in the hypothalamus of lactating goats was found to block milk production and to produce a type of diabetes insipidus characterized by delayed onset and absence of the normal interphase (GALE and LARSSON 1963) That insufficiency in secretion of ACTH TSH, and STH (somatotrophin) from the anterior pituitary lobe caused the block in milk production was suggested by the ability of replacement therapy to restore lactation in goats with brain lesions In contrast no inadequacy in secretion of prolactin (LTH) a hormone essential for milk synthesis in goats (COWIE and TINDAL 1962), could be shown to contribute to the decline in milk synthesis Support for the hypothesis of central inhibitory control of prolactin secretion was ascribed to these results

The delayed onset and absence of the normal interphase in the diabetes insipidus manifested by goats with median eminence radiolesions was tentatively attributed to the very slow rate of destruction of the supraoptico-hypophysial tracts and hence the very gradual release of antidiuretic hormone (ADH) from the slowly degenerating neural lobe In contrast diabetes insipidus marked by an abrupt onset and a well-defined normal interphase is produced by methods which cause a rapid destruction of the supraoptico-hypophysial tracts and of the neural lobe e g electrolytic lesions (FISHER INGRAM and RANSON 1935) and pituitary stalk section O CONNER 1952) Such procedures cause the sudden release of large amounts of stored ADH from the neural lobe

In the present study the median eminence region in four lactating goats was coagulated by radio frequency (RF) current in order to investigate further (1) the role of the central nervous system in regulating the secretion of adenohypophysial hormones essential for lactation and (2) the type of diabetes insipidus produced by abrupt destruction of the supraoptico-hypophysial tracts in this species

Methods

General care of the animals

The four adult, lactating goats used in this study were maintained in metabolism cages on a daily ration of 250 g of grain with 4 g of NaCl added Water and hay were available ad libitum. Water intake volume and specific gravity of urine and rectal temperatures were recorded daily Average daily room temperature ranged from 17 to 20 C.

Lactation measurements

During this study which was begun in March and continued through September 1962 milk production was measured after regular twice-daily milkings (at 0930 and 1700) with particular care being taken to empty the udder completely by massage Occasionally the specific gravity of milk samples cooled to room temperature was measured and milk fat determined by the method of LINDSTROM (cf BARTEL 1928) Na^+ and K^+ concentration in milk and urine samples were occasionally determined on an EEL Flamephotometer while CL^- concentration was measured according to BRUN (1949)

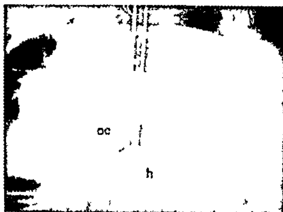


Fig 1 *An X ray picture of localization of electrodes for production of radio frequency lesions in the hypothalamus of a goat* This sagittal view shows the tips of the two pairs of electrodes positioned in the median eminence region of Goat A oc = optic chiasm h = hypophysis

Drinking pattern

The drinking pattern of goats before and after production of hypothalamic lesions was determined by means of a kymograph which continuously recorded the weight of the drinking bucket

Replacement therapy

Subcutaneous injections into the neck region of varied combinations of the following hormones were given to goats at times during the study (daily dosage) prolactin (luteotrophin LTH) NIH P S 3 Ovine[1] 300 I U somatotrophin (STH growth hormone) NIH GH S 4 Ovine[1] 12 mg ACTH Schering Corporation ACTH Depot 3 I U triiodothyronine (T3) Smith Kline and French[2] 0 7 mg and protomine zinc insulin Vitrum 8 I U

Production of radio frequency lesions in the hypothalamus

Under general anesthesia one pair of electrodes (in Goats C and D) or two pairs (in Goats A and B) were implanted bilaterally in the median eminence region by use of a Horsley Clarke stereotaxic instrument modified for use with the goat and by X ray control (Fig 1) A specially designed head holder which gripped the goat s horns fixed its head rigidly during operation The electrodes which were fixed permanently to the skull surface with dental cement were constructed of steel cannulas 1 mm in diameter with thermocouples inserted in the bore and were insulated except for 2 or 3 mm of the tip By this arrangement it was possible to measure the temperature of the brain tissue at the electrode tips Hypothalamic lesions were produced by passing radio frequency energy between the electrodes generated by a converted 30 W crystal controlled U S Army surplus BC 604 transmitter All speech and modulation circuits were removed from the transmitter and a power supply added The output power was regulated by varying the screen grid voltage of the final power amplifying stage The screen grid voltage was supplied by a cathode follower with the DC grid voltage set by a potentiometer The transmitter 30 Ω output impedance was matched by a quarter wave transformer to a 150 Ω twin lead feeder which carried the RF energy to

[1] The prolactin and the somatotrophin were a gift of the Endocrine Study Section National Institutes of Health U S P H S

[2] The triiodothyronine was a gift from Smith Kline and French Co

Table I The effect of radio frequency hypothalamic lesions in lactating goats

Goat	Date of lesion	Location of lesion	Lactation (per cent of pre lesion level)		Diabetes In sipidus	Organ weights (g)	
			After lesions	After hormonal therapy		Ovaries	Uterus
A	4/62	Bilaterally in median eminence basal hypothalamus optic chiasm	20	95	+++	1 9	11 9
B	5/62	Bilaterally in median eminence basal hypothalamus	30	100	++	2 4	14 0
C	6/62	Bilaterally in median eminence	15	130	+		
D	8/62	Unilaterally in median eminence	80	–	[1]+–	3 4	82 0

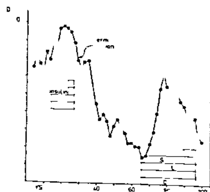
[1] In Goat D the acute phase of diabetes insipidus occurred but the permanent phase of elevated water turnover failed to develop

a matching transformer on the head of the animal The matching transformer was found necessary because of the large capacitive component of the electrode impedance

In Goats A and B hypothalamic lesions were produced under general anesthesia y following implantation of the two pairs of electrodes In Goat A a large lesion in the basal hypothalamus was made by passing RF energy alternately between pairs of electrodes i e laterally diagonally and front to back Current was applied sufficient to raise the temperature at the electrode tips to 60 to 70 C for 4 min with each passage In Goat B a smaller hypothalamic lesion was made by raising the temperature to 65 for 3 min during passage of RF energy in the same manner between alternate pairs of the four electrodes In Goats C and D the RF lesions were made several weeks after electrode implantation and while the animals were standing in their normal environment in metabolism cages In Goat C the temperature at the tips of the electrode pair was raised to 65 for 4 min During this hypothalamic heating the goat gave no indication of pain or distress In Goat D hypothalamic temperature was elevated to 75 for 7 min This animal also failed to show any obvious signs of discomfort of considerable interes was the observation that it drank water during this brief period of central hea ing a finding in accord with the thermostatic theory of water regulation (ANDERSSON and LARSSON 1961 ANDERSSON GALE and SUNDSTEN 1962) Development of lesions in the supraoptico-hypophysial tracts one to three days post heating was indicated by the onset of diabetes insipidus

Local cooling performed in the hypothalamus

In goat C local cooling of the preoptic/anterior hypothalamic region was performed for 8-hour periods on 2 successive days before production of lesions By means of rapid ice water perfusion of a silver thermode implanted in the brain several months earlier the brain temperature recorded 5 mm lateral to the thermode by a needle applicator



F g 2 A

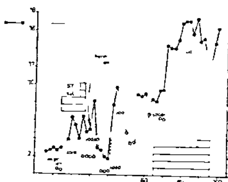


Fig 2 B

F g 2 A *Effect of hormonal replacement on milk production in Goat A before and after hypothalamic lesions* Prior to lesions daily administrat on of ACTH (3 I U) and T3 (0 7 mg) caused a 30 per cent increase in milk production Addition daily of STH (1? mg) and insulin (8 I U) to this regimen failed to stimulate lactation further Product on of hypothalam c lesions by RF heating resulted in a p ompt decline in milk synthesis and the onset of diabetes insipidus (see Fig 2 B) Daily admin strat on of STH insulin T3 and ACTH rapidly resto ed the depressed milk production but deterioration in the health of the animal during replacement apparently caused lactation to decline again Addition of prolactin (LTH) (300 I U daily) to the therapeutic regimen failed to imp ove lactat on further The animal was found dead on the day indicated by the cross

Fig 2 B *Pr duction of diabetes insip dus by hypothalamic les ons in Go t A — elevated water turnover augmented by hormonal replacement* Administration of ACTH and T3 befo e RF heating caused a 100 per cent increase in water turnover but addition of STH and insulin to this therapy failed to elevate water metabolism further Later destruction of the median eminence by RF coagulation induced diabetes ins p dus of the classical type *i e* characterized by (1) the abrupt onset of a b ief period of polydipsia and polyur a followed by (2) a well-defined normal nter phase of 7 days during which water turnover returned to pre lesion lev ls and then (3) the onset of the permanent phase of polydipsia and polyuria When eplacement with STH in sul n T3 and ACTH was given the existent high level of water turno er was elevated 100 per cent furth r Addit on of prolactin (LTH) to this regimen caused some amelioration of diabetes ins pidus

was lowered to 35 during cooling The construction of the silver thermode the technique of implantation and of perfusion and effect of chronic central cooling on alimentation thermoregulation and thyroid activation have been reported earlier (ANDERSSON and LARSSON 1961 ANDERSSON GALE and SUNDSTEN 1962)

Histology

The goats were sacrificed by decapitation under Nembutal anesthesia with the exception of Goat A which died during the study The carotids were rapidly perfused with physiological saline followed by 5 per cent formol saline Brains were removed with pituitaries attached and fixed in 5 per cent formol saline for several weeks A block of tissue containing the diencephalon was then dissected out embedded in celloidin and cut in transverse sections 35 μ thick. Alternate sections were stained with toluidine blue and with hematoxylin (LOYEZ cf CULLING 1957) Pituitary glands were embedded in paraffin cut serially at 15 μ and stained according to LADEWIG s (1938) modification of MALLORY Ovaries and uteri were removed weighed to 0 1 g fixed in 5 per cent formol saline embedded in paraffin cut at 15 μ and stained with hematoxylin and eosin

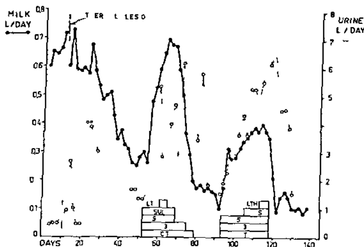


Fig 3 Block of lactation and onset of diabetes insipidus after hypothalamic lesions in Goat B Milk production restored by hormonal therapy Following RF destruction of the med an eminence there occurred a decline in lactation and an onset of diabetes insip dus of the classical type Note that the fall in milk production is paralleled by an amelioration of diabetes ins pidus Following administration of insulin STH T3 and ACTH milk synthesis was promptly restored to the pre lesion level while diabetes insip dus was increased to a level even greater than that seen post lesion The brief injection of prolactin (LTH) is not believed to have increased milk yield further Upon withdrawal of insulin and STH from the therapy milk yield fell sharply whereas diabetes insipidus was maintained at the same high level until withdrawal of T3 and ACTH A second replacement course failed to restore milk synthesis to more than 60 per cent of pre lesion values whereas water intake was elevated to the same high levels seen during the previous therapeutic treatment

Results

1 Block of lactation following radio frequency lesions in the hypothalamus restored by hormonal replacement

Within several days after the production of hypothalamic lesions by radio frequency warming there occurred in 3 of 4 goats a decline in lactation which within 3 to 7 weeks had fallen to 15 to 30 % of pre lesion levels (Fig 2A 3 and 4 Table I) Replacement with varying combinations of hormones was then administered with attention being given to evaluating the importance of hormones was then administered with attention being given to evaluating the importance of prolactin (LTH) in restoring the depressed milk yield Within 10 to 20 days following the daily administration of ACTH STH T3 and insulin milk production had risen to or near pre lesion levels The addition of prolactin to this therapeutic regimen could not be shown to improve milk yield further nor could its withdrawal be shown to reduce it

Goat A incurred a particularly large lesion in the basal hypothalamus Failure of hormonal replacement to maintain the high level of lactation initially

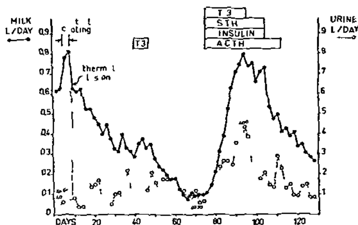


Fig 4 Complete restoration of lactation in Goat C with hypothalamic lesions by hormonal replacement excluding prolactin Local cooling of the preoptic/anterior hypothalamic region (the heat loss center) for two consecutive days evoked a 30 per cent increase in milk yield apparently due to stimulation of secretion of pituitary TSH (thyroid stimulating hormone) and possibly other hormones (ANDERSSON *et al* 1963) Following RF lesioning of the median eminence the marked *decline in milk production was only slightly checked by administration of T3 for 7 days* In contrast, replacement daily with T3 STH insulin and ACTH induced a rapid restoration of milk synthesis to the maximum level attained during brain cooling i e 30 % above pre lesion control levels The effect of selective withdrawal of hormones from the replacement suggests the importance of T3 and STH and to a lesser degree ACTH in restoring milk production The moderate diabetes insipidus occurring after lesions was augmented considerably by therapy

attained (Fig 2A) is believed to be related to the extensive brain damage Reduction in food intake partial blindness and a general deterioration in health were observed in this animal during the latter part of the replacement period

In Goat B replacement therapy afforded a rapid restoration of milk production to pre lesion levels (Fig 3) The addition of prolactin to the therapeutic regimen for a brief period is not believed to have benefitted lactation further since its inclusion occurred when milk yield was nearly maximally restored and its withdrawal was not associated with a decline in milk yield An attempt to restore milk production a second time after stopping all replacement therapy had depressed lactation was only partially successful Failure in this instance to achieve more than 60 per cent restoration is unexplained but may be due to formation of antibodies against the protein hormones injected previously

Strikingly the best restoration of lactation occurred in Goat C which was not given any prolactin at all in the hormonal supplement (Fig 4) In this animal administration of T3 for seven days during the declining phase of lactation (after lesions) afforded very slight benefit showing that other hormones were essential for restoring milk production That median eminence

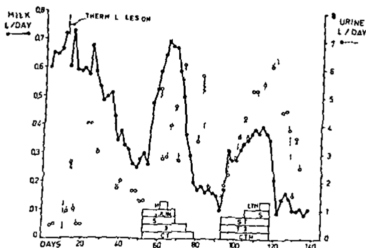


Fig 3 *Block of lactation and onset of diabetes insipidus after hypothalamic lesions in Goat B Milk pro duction restored by hormonal therapy* Following RF destruction of the median eminence there oc curred a decline in lactation and an onset of diabetes insipidus of the classical type Note that the fall in milk production is paralleled by an amelioration of diabetes insip dus Following administration of insulin STH T3 and ACTH milk synthesis was promptly restored to the pre lesion level while diabetes insipidus was increased to a level even greater than that seen post lesion The brief injection of prolactin (LTH) is not believed to have increased milk yield further Upon withdrawal of insulin and STH from the therapy milk yield fell sharply whereas diabetes insipidus was ma ntained at the same high level until withdrawal of T3 and ACTH A second replacement course failed to restore milk synthesis to more than 60 per cent of pre lesion values whereas water intake was elevated to the same high levels seen during the pre vious therapeutic treatment.

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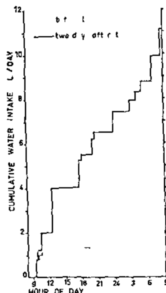


Fig 6 Drinkin pattern b fore and after induction f diabetes in sip dus by hyphothalami l sions in Goat B Prior to the destruction of the median eminence by RF heating this animal normally drank pract cally all of its 24 hour water requirement w thin several hours after eating its morning food ration In contrast after les ons it ingested large amounts of water continuou ly throughout the 24 hour period—except du ing the normal interphase when it reverted to the pre lesion pattern

3 Production of diabetes insipidus by RF lesions in the hypothalamus

Within one to three days after RF heating in the hypothalamus diabetes insipidus developed in all 4 goats In the acute phase which lasted 3—5 days water intake and urine output increased 3—10 fold with the specific gravity of the urine falling to very low values (Fig 2B 3 4 and 5) A well demarcated normal interphase then became manifest for 4—8 days during this interval urinary output fell to very low amounts and of a high specific gravity while water intake as well was reduced to or below pre lesion levels The permanent phase of diabetes insipidus then supervened in 3 of the 4 animals with the return of marked polydipsia and polyuria After 4—5 weeks the severity of diabetes insipidus tended to decline Of interest was the observation that this amelioration of the elevated water turnover generally paralleled the fall in milk production Goat D after displaying an initial onset of diabetes insipidus after lesions failed to develop the permanent phase of polyuria (Fig 5) — this failure apparently resulting from the incompleteness of the median eminence lesion

4 Pattern of water intake after RF hypothalamic lesions

The drinking pattern of normal goats and of those with diabetes insipidus after RF hypothalamic lesions was studied by means of a kymograph which continuously recorded the weight of the drinking buckets Before lesions two goats drank practically all of their daily water requirement within 2—3 hours after eating their morning ration of grain In contrast after median eminence

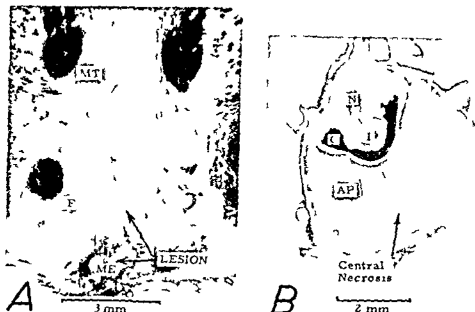


Fig 7 Photomicrographs shown (A) the location of RF le ions in the hypothalamus of Goat B and (B) the effect of hypothalamic lesions on the hypophysis of this animal

A Bilateral destruction of the median emin nce (ME) d generation of one column of fornix (F) and disruption of the normal configurat on of the third ventricle are shown Stain Loyez

Shown are the large area of central infarction in the adenohypophysis (AP atrophic change the neural lobe (N) a id absence of marked change in the intermediate lobe (I) Colloid st (C) Stain Ladew g

lesions, these goats displayed a pattern of continuous drinking throughout the 24 hour period (Fig 6) — except durin, the normal interphase when the pre lesion pattern returned

5 *Histology*

Brains of Goats A B and C exhibited lesions in the midline of the basal hypothalamus causing bilateral destruction of the median eminence while the brain of Goat D showed incomplete destruction of the median eminence In *Goat A which died during the study the rostral extent of the lesion transected* the optic chiasm and continued caudally into the mammillary bodies Extensive damage dorsal to the median eminence obliterated the third ventricle Goat B was found to bear a lesion extending from the optic chiasm to the mammillary bodies and causing damage to the third ventricle and to one column of the fornix (Fig 7A) In Goat C the lesion destroyed the median eminence at the level of the separation of the stalk Goat D which failed to show lactational block and only brief elevation of water turnover showed in

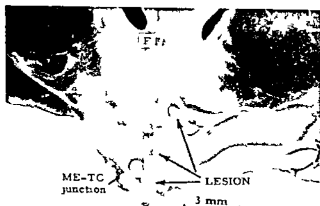


Fig 8 *I complete destruction of the median eminence after hypothalamic lesions in G at D* The RF lesion at its broadest extent is illustrated in this photomicrograph of a transverse sect on of the hypothalamus Note that a port on of the junct on of the median eminence (ME) with the tuber cinereum (TC) remains intact and that the lesion is largely confined to one s de of the hypothalamus Lactation in this an mal was only slightly impaired and after the initial onset dia betes insipidus abated spontaneously Stain Loyez

complete destruction of the median eminence the lesion being limited to one half of the basal hypothalamus (Fig 8)

The pituitary glands of 3 goats developing lactation block displayed large areas of central necrosis in the anterior lobe (Fig 7B) although considerable amounts of apparently well vascularized parenchyma were present especially in the posterior portion of the adenohypophysis Infarction in the anterior lobe of Goat D which failed to exhibit impairment in lactation was less extensive than in the other animals The neural lobes of these animals showed atrophic changes while no gross alterations were observed in the intermediate lobes

Discussion

The present results clearly indicate that prolactin (LTH) is not an essential component of the hormonal regimen required to restore the depressed milk production in goats with median eminence lesions Since prolactin in large amounts is indeed essential for milk synthesis in the goat (Cowie and Tindal 1961 Cowie and Tindal cf Folley 1961) these data indicate that secretion of this adenohypophysial hormone can continue unabated after the pituitary gland has been denervated from central nervous control It can not be ruled out however that concurrent administration of permissive hormones such as ACTH TSH and insulin may be required for maximal secretion of prolactin from the denervated hypophysis Although it has been recently suggested that in the rat progesterone may stimulate prolactin (luteotrophin) release during pseudopregnancy via positive feed back control (Rothchild 1962) the impor

tance of this mechanism is difficult to assess since ovariectomy fails to inhibit lactation (and hence prolactin secretion) in this species (FOLLEY 1956 GALE 1960) Similarly, estrogen administration has been shown to increase prolactin content in the adenophypophysis in intact rats (MEITES 1961) and to stimulate both synthesis and release of prolactin in anterior lobes cultured *in vitro* (NICOLL and MEITES 1962), but the failure of ovariectomy to impair lactation indicates that the stimulating action of this steroid is not essential for the secretion of physiological amounts of prolactin SAWYER (1962) has recently reported that whereas inplantation of estrogen crystals in the basal hypothalamus of rabbits causes an increase prolactin content of the pituitary the production of electro lytic lesions in this area depletes the hypophysis of its prolactin content and promotes milk secretion At present therefore it appears that although some of the endocrine factors which play a stimulatory role in prolactin synthesis are under central excitatory dominance *e g* estrogen cortisol (HARRIS 1955) the primary control of prolactin is exerted via central tonic inhibition Additional evidence in support of the hypothesis of inhibitory regulation of prolactin and data tending to disprove the theory that oxytocin stimulates prolactin secretion via direct action on the acidophil cells have been discussed in a preceding communication (GALE and LARSSON 1963)

In goats with median eminence lesions produced rapidly by RF coagulation the presence of large central infarcts in the anterior pituitary lobes is in contrast to the relative lack of adenohypophysial scarification seen in goats in which lesions had been induced gradually by proton radiation (GALE and ⸺ 1963) It is remarkable that despite the extensive necrosis seen in the pituitary lobes in the present study they were still capable of secreting he large amounts of prolactin required during lactation — as shown by the lactational restoration afforded by replacement therapy excluding prolactin It is possible that the somewhat greater decline in lactation observed in goats with RF coagulation than in those with proton radiolesions may have been due in part to the greater degree of non specific pituitary depression secondary to abrupt interruption of the hypophysial portal vessel supply (DANIEL and PRICHARD 1958) The slower rate of hypothalamic destruction caused by proton radiation may have allowed time for some compensatory vascular adjustment perhaps via the short portal vessels from the neural lobe and thus minimized ischemic necrosis In any case the results of these studies involving both types of lesions suggest that the stoppage of milk synthesis was primarily due to blockade of central stimuli which regulate secretion of lactogenic hormones It is well known that lesions destroying the median eminence comprising as it does the final common path between the brain and the adenohypophy is interfer with secretion of ACTH (McCANN 1953 and many others) and of TSH (most recently demonstrated in the goat ANDERSSON *et al* 1963) Although the nature of the regulation of STH is less well understood (REICHLIN 1960) the present results are at least suggestive of central nervous participation That

oxytocin deficiency in goats with median eminence lesions was not a cause in the decline in milk production secondary to incomplete removal of milk was shown in a previous study (GALE and LARSSON 1963)

The present production in goats by RF coagulative lesions of the classical diabetic syndrome of FISHER INGRAM and RANSON (1935) — characterized by a rapid post lesion elevation in water turnover followed by a normal inter phase and then by a permanent phase of polyuria polydipsia — is in contrast to the unusual type of diabetes insipidus evoked by proton radiolesions (GALE and LARSSON 1963) In this latter instance there was observed a delay of 30 days post radiation in the onset of diabetes insipidus which further failed to exhibit the normal interphase It appeared that the permanent phase of dia betes insipidus came on directly without the manifestation of the earlier phases It was postulated that this unusual diabetic syndrome was due to the very slow severance of the supraoptico hypophysial tracts by the gradually enlarging radiolesions and hence to the continuous liberation of small amounts of antidiuretic hormone from the neural lobe as the axons degenerated Accord ingly therefore such goats bearing proton radiolesions would maintain normal water balance until total depletion of hormone had occurred at which time the permanent phase of diabetes insipidus would become manifest The present finding that rapid coagulative lesions induced the classical tri phasic diabetic syndrome thus provides correlative evidence in support of this interpretation According to O CONNER (1952) the acute phase and the normal interphase are due respectively to (1) the abrupt denervation of the neural lobe by destruction of the supraoptico hypophysial tracts which would prevent release of antidiuretic hormone in response to physiological demands and (2) the sudden release of large stores of hormone as the neurohypophysial axons de generate *en masse* That considerable amounts of antidiuretic hormone are normally stored in the neural lobe of the goat was indicated both in the present study by the well defined normal interphase lasting four to eight days and also in goats subjected to proton irradiation by the 30 day delay in onset of the permanent phase of diabetes insipidus In contrast albino rats apparently have relatively smaller stores of antidiuretic hormone in the neurohypophysis since rats made diabetic by hypothalamic electrolytic lesions exhibit a normal interphase of shorter duration (two to four days) and during which water turnover does not return to normal levels Rather water intake remains three to five times greater than normal during this short period although it is re duced about 50 per cent from the maximum of the acute phase (GALE TALEIS NIK and McCANN 1961) Thus it appears that less antidiuretic hormone is normally stored in the rat neurohypophysis than in that of the goat the cat (FISHER INGRAM and RANSON 1935) and the dog (O CONNER 1952)

Since the decline in milk production in goats with lesions was paralleled by an amelioration of the diabetes insipidus and since hormonal replacement which restored milk production also reestablished the previous high level of

water turnover, it appears that some pituitary hormones essential for milk synthesis are also required for full manifestation of diabetes insipidus ACTH and T3 were most effective in elevating water metabolism In contrast, in albino rats in which lesion induced diabetes insipidus had been attenuated by prior surgical hypophysectomy, the injection of ACTH but not of STH or of T3 was found to elevate water turnover (GALE TALEISNIK, and MCCANN 1961) Since neither STH nor thyroid hormone have been found to be essential for restoration of lactation in rats subjected to hypothalamic lesions (GALE *et al* 1961) or hypophysectomy (COWIE 1957 BINTARNINGSIH *et al* 1958) whereas ACTH (or adrenal corticoids) play a critical role it appears that ACTH exerts a lactogenic stimulus in the rodent which is shared with STH and TSH in higher mammals

The 30 per cent augmentation in milk yield resulting from chronic local cooling in the preoptic/anterior hypothalamic heat loss center in the present study was apparently the result of three factors (1) stimulation of secretion of TSH and perhaps other galactopoietic hormones (ANDERSSON *et al* 1962 ANDERSSON *et al* 1963), (2) increased food intake (ANDERSSON and LARSSON 1961) and (3) chronic elevation of core temperature to fever levels during cooling which must have increased metabolism by a Q_{10} effect (ANDERSSON, GALE and SUNDSTEN 1962) Thus although lactation and adaptation to cold stress appear superficially as dissimilar biologic phenomena they share a common reliance upon interaction between the central nervous and endocrine systems for appropriate metabolic stimulation

The author wishes to express thanks to Associate Professor BENGT ANDERSSON for his helpful advice and encouragement during this investigation

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Handling of Glycerol in the Kidney

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Abstract

KRUHOFFER P and O I NISSEN *Handling of glycerol in the kidney* Acta physiol scand 1963 59 284—294 — Essentially complete reabsorption of glycerol (and urine/plasma ratios below one) was observed in cats at low filtered loads With increasing filtered loads the reabsorption rate continued to rise and the reabsorption fraction converged upon a value of about 0 4 The rate of metabolic conversion of glycerol in the kidneys — simultaneously determined from measurements of renal blood flow and arterio venous deficits — exceeded the reabsorption rate at low filtered loads whereas the relation was undoubtedly reversed at high filtered loads Stop flow experiments indicated that the most distal parts of the nephron are sparsely permeable to glycerol and that concentrations below that of arterial plasma may arise in proximal parts The observations may be accounted for on the assumptions that reabsorption at low filtered loads is predominantly a conversion reabsorption (diffusion from the tubular lumen into tubular cells maintained by a metabolic conversion inside the cytoplasm) and that at high filtered load well above the level of saturation of the glycerol converting enzymes it is essentially due to transcellular back diffusion through the (proximal) tubular cells The pattern of renal excretion of glycerol may thus be interpreted without resorting to special transmembrane transfer mechanisms

It is generally conceded that the excretion of a great variety of substances by the mammalian kidney can be adequately accounted for by glomerular ultrafiltration followed by tubular reabsorption Usually two types of reabsorption processes are considered passive transcellular back diffusion caused by a urine to blood concentration gradient set up by tubular reabsorption of water and active transcellular transfer involving a temporary combination

of the substance in question with a cellular carrier element (Transfer of ions downhill an electrochemical gradient is neglected here since this article deals with the renal handling of a non electrolyte)

In the case of urea and of ethanol tubular reabsorption is widely believed to be exclusively a matter of transcellular back diffusion Accordingly the reab sorption fraction of these substances is (widely) independent of the plasma concentration but dependent on the reabsorption fraction for water Due to a rather low permeability of the tubular cells to urea the reabsorption fraction for this substance ordinarily amounts to only about 0 4 In the case of ethanol — to which the tubular cells are highly permeable — the reabsorption fraction is closely similar to that of water and thus ordinarily close to one

In other cases the reabsorption may be exclusively effected by an active transfer system of limited transfer capacity The rate of reabsorption will then rise to a maximum value with increasing filtered loads and the reabsorption fraction will decrease from essentially one towards zero Glucose is generally held out as a prototype of this category

It appears to the authors that processes other than the two just mentioned (or a combination of these) may be involved in tubular reabsorption Kidney tissue is known to metabolize a number of organic substances which are also known to be subject to tubular reabsorption (lactate ketone bodies glycerol amino acids etc) In such cases a metabolic conversion of the substance might keep its intracellular concentration at a low level and thus give rise to a diffusion of the substance from the tubular lumen into a tubular cell the conversion product(s) would subsequently be delivered to the blood or to the urine

The main purpose of the work reported in this paper has been to investigate whether such a conversion reabsorption might play a role in the reabsorption of glycerol With this in view the rate of reabsorption of glycerol in the kidneys of cats was determined at various plasma concentration levels and compared with the rate of metabolic conversion of glycerol in the kidneys simultaneously determined (as the product of the renal blood flow and the renal arteriovenous glycerol deficit minus the rate of urinary excretion of glycerol)

Methods

All experiments were performed in nembutal anesthetized cats weighing about 4—5 kg The trachea a carotid artery and a jugular vein were cannulated the last serving for infusions

In 5 successful experiments simultaneous determinations were made of the rate of metabolic conversion and the rate of reabsorption of glycerol in the kidneys whereas only the latter was determined in some other experiments Constant levels of glycerol in arterial whole blood and plasma were obtained by continuous infusion of glycerol solutions for at least 30 min (following a priming dose) the rate of infusion of glycerol varied from approximately 96 mg per hour and kg to 454 mg per hour and kg correspond ing to arterial plasma concentration levels ranging from approximately 5 2 mg per cent

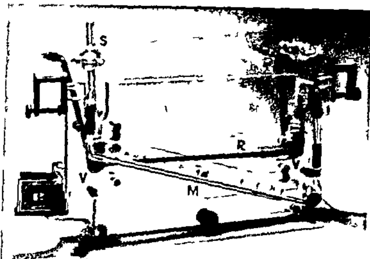


Fig 1 *Differential pressure flowmeter* R Resistance glass tube V Glass vials through which the blood enters and leaves the resistance tube M Manometer S Stopcocks used for zero setting of the manometer For details confer the text

to 111 mg per cent At the lower concentration levels urine flow rates (0 05—0 3 ml/min) adequate for clearance determinations were evoked by infusion of 2 per cent NaCl solution or 1—3 per cent Na_2SO_4 solution

A continuous infusion of heparinized blood from a donor cat was maintained through the experiment to compensate for the blood samples drawn and for a small bleeding and at high urine flow rates a saline water mixture was infused at a rate sufficient prevent dehydration Inulin administered by continuous infusion was employed for determination of the glomerular filtration rate Arterial and renal venous blood for determination of inulin glycerol (and in some cases PAH) was sampled over a few minutes slightly prior to the middle of the urine collection periods The duration of these periods varied from 9 to 17 min depending upon the level of the plasma glycerol concentration and the diuresis 3—4 periods were used in experiments with determinations of the metabolic conversion whereas 5 were ordinarily used when only the reabsorption was studied Urine was collected from a uretheral catheter inserted via a temporary opening in the apex of the bladder the expanded end of the catheter was fixed in the trigonum by means of a ligature around the urethra Emptying of the bladder which remained exposed in the midline incision was brought about by manual compression

The rate of blood flow through the kidneys was determined in heparinized cats by shunting the renal venous blood through a glass tube resistance and measuring the fall in pressure over the resistance. A special technique was developed which permitted connection with flowmeter to be established without interrupting the blood flow through the kidneys

The construction of the differential pressure flowmeter appears from Fig 1 A straight glass tube (R) about 20 cm long and with a bore of 3 4 mm was used as the resistance The blood entered and left the tube through the lower part of small vertical glass vials (1 1/2 × 3 1/2 cm) (V) In the vials the blood was covered by a layer of paraffin oil The air in the upper end of the vials was connected to the ends of a manometer (M)

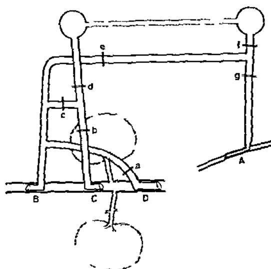


Fig 2 Diagram illustrating the connections between the differential-pressure-flowmeter and the vascular system For details confer the text

by way of ground glass joints Connection could also — for zero setting of the manometer — be established to the atmosphere by stopcocks (S) The manometer was V shaped one branch was expanded to serve as manometer fluid reservoir and the other — the measuring branch — had a slope of approximately 16 degrees towards the horizontal and rested on a millimeter scale Coloured kerosine was used as manometer fluid The apparatus was mounted on a lucite plate and during the experiment and the calibration it was submerged in water at 37 ± 0 05 C. With blood of ordinary composition a deflection of about 100 mm (corresponding to about 23 mm of vertical water column) was obtained at a flow rate of 100 ml per minute All glass parts contacted by blood were siliconized glass parts and cannulae were connected by PVC tubes of 5 mm bore

The flowmeter — prefilled with heparinized donor blood or saline — was connected to the venous system as indicated in the diagram Fig 2 The first step — in order to have only the venous blood from the kidneys passing through the flowmeter — was to ligate all branches of the renal veins (spermatic adrenal etc) and of an approximately 5 cm piece of the posterior caval vein distal to the renal veins With the tubing clamped at points a—g cannula A was inserted into a jugular vein Cannula B was inserted into the posterior caval vein after ligation immediately proximal to the site of insertion (a bulldog clamp was temporarily placed on the vein distal to the site of insertion) Unclamping at e and g then allowed blood from the hind limbs to pass into the jugular vein The next step was the insertion of cannula C into the posterior caval vein distal to the origin of the renal veins followed by unclamping at b c, d and f Subsequently the caval vein was ligated immediately proximal to the origin of the renal veins (often a vein from the right adrenal gland also had to be ligated to allow the last cannulation to be carried out without bleeding) Cannula D was then inserted into the caval vein proximal to the renal veins with a bulldog clamp temporarily applied proximal to the site of insertion Subsequent unclamping at a and clamping at c allowed the blood from the hind limbs to pass from B to D while the blood from the kidneys would enter the flowmeter via C and return to the body via A and D Samples of mixed renal venous

blood were drawn from the "exit side" of the flowmeter The zero flow position of the manometer fluid could be tested during the experiment without interfering with the renal blood flow — by unclamping the tubing at c and clamping at d and f

At the end of the experiment the flowmeter was calibrated For this purpose some 80—100 ml of blood were collected from the arterial cannula and (generally by removal of some plasma) the hematocrit was adjusted to a level close to the average of those observed in the experimental periods This blood was then run through the termostatized flowmeter from a burette with a heating mantle A constant rate of flow during each calibration run was achieved by keeping the blood surface level in the burette constant (by elevating the burette mounted in screw stand) Recording the time spent by the blood surface in passing from one burette mark to another (50 ml — or 40 ml apart) allowed calculation of the rate of flow The flow rate versus pressure fall relation proved to be essentially rectilinear As might be expected the pressure fall decreased with decreasing hematocrit values at a given rate of flow but it turned out that over a range of hematocrit values from 26 to 46 per cent the flow rates corresponding to a particular pressure drop were nearly inversely proportional to the hematocrit value Consequently the rate of flow (f_p) in an experimental period with a hematocrit of ht_p was calculated as $f_p = f \times ht_c/ht_p$ where ht_c is the hematocrit of the blood used for calibration and f is the flow rate read from the calibration chart at the pressure drop prevailing (on an average) during the experimental period The hematocrit corrections were generally below 5 per cent and only exceeded 10 per cent in one experiment During each experimental period manometer readings were taken at 1/2 min intervals and the average was used for calculation of the flow rate It is estimated that the overall error of the determination of the rate of blood flow is below 5 per cent

A few *stop-flow experiments* were performed A small polyethylene catheter was inserted in one ureter close to the renal pelvis Stops lasting from 10—11 min were made during mannitol diuresis About 30 successive effluents of 150—250 μl were collected over a [period] of about 2—3 min

Glycerol was determined in whole arterial and renal venous blood and arterial plasma by an enzymatic method being a modification of that of WIELAND (1957 a) 400 μl of blood or plasma (and water and standard solution) was deproteinized by addition of 1 000 μl of 6 per cent (W/V) perchloric acid and careful shaking After centrifugation at 4 500 rpm for 15 min 800 μl of supernatant were mixed with 50 μl of 6 8 N KOH (to precipitate perchlorate as the potassium salt) Upon centrifugation 200 μl of supernatant were transferred to 2 ml of a mixture made by adding 0 65 mg DPN Boehringer 1 4 mg ATP Boehringer 10 μg glycerophosphate dehydrogenase Boehringer and 10 μg glycerokinase Boehringer to each 2 ml of the following buffer mixture 20 8 g hydrazine hydrate (24 per cent) 1 5 g glycine 0 2 ml of 0 2 M $MgCl_2$ 0 8 ml of conc HCl and approximately 2 ml 6 8 N KOH made up with water to 100 ml (and pH adjusted to ca 9 4) Upon incubation for 75 min at room temperature the density at 340 mμ was determined in silica cuvettes of 1 cm layer thickness As given above concentrations up to about 40 mg per cent can be measured (concentration density proportionality) Above this concentration some part of the supernatant from the last precipitation was replaced by supernatant from a blank (water) precipitation At blood concentrations at about 20 mg per cent the standard deviation was found to be 1 0 per cent of the mean

Inulin was determined by the method of BOJESEN (1952) using 1/5 the volumes of the original procedure

PAH was determined in whole blood by the method of SMITH et al (1945) using a 1 15 dilution during the cadmium precipitation The recovery was on an average 81 per cent

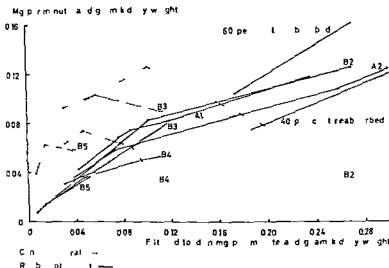


Fig 3 *The relationship between filtered glycerol loads and the rates of glycerol reabsorption and metabolic conversion* A filtered load of 0 1 mg/min/g corresponds to a plasma glycerol concentration of roughly 35 mg /

Results

In 5 exp the rate of blood flow through the kidneys was on an average 94 ml/min (range 53—139 ml/min) and the filtration rate on an average 12 4 ml/min (range 5 9—21 8 ml/min) In 6 other exp where the renal blood flow was not determined the filtration rate was on an average 13 7 ml/min (range 5 0—22 8 ml/min) Thus the procedure of the flow measurement did not seem to have any great effect on kidney function in this context it may also be noted that the rate of urine flow was not significantly altered by the procedure In 4 exp the rate of blood flow was determined by means of PAH (rate of urinary excretion/arteriovenous deficit) as well as by means of the flowmeter In these and similar experiments undertaken to study the handling of ketone bodies in the kidney good agreement was generally obtained between the two types of measurement but discrepancies of about 20 per cent were occasionally observed in these instances the PAH determinations showed greater variations from one period to another than did the flowmeter determinations

At increasing levels of the plasma glycerol concentration and the filtered load the reabsorption fraction (fraction of the amount filtered being reabsorbed) decreased from values close to one down to values of about 0 5 and at the highest filtered loads the reabsorption fraction appeared to converge towards a limiting value of about 0 4 (cf Fig 3) In other words it appeared that the absolute rate of glycerol reabsorption was steadily increasing with rising levels of the filtered load

In 4 exp at plasma glycerol concentrations in the range of 5 2—8 6 mg per cent, lower glycerol concentrations were observed in the urine than in the arterial blood plasma The lowest observed U/P ratio was 0 37

In the 5 exp in which the glycerol concentrations of arterial and renal venous whole blood were determined the renal extraction fraction for glycerol was found to decrease at increasing levels of the arterial blood concentration The highest extraction fraction observed was 0 56 (at 7 mg per cent) and the lowest 0 053 (at 105 mg per cent)

The rate of renal conversion of glycerol (calculated as the arteriovenous concentration difference times the rate of renal blood flow minus the rate of urinary excretion) increased with increasing arterial blood concentrations from the lowest levels studied (5—8 mg per cent) up to about 15—25 mg per cent (corresponding to filtered loads of about 0 05 mg per minute and gram kidney in Fig 3) There appeared to be a flat maximum at this blood concentration level, but the fact that the rate of conversion was found to decrease appreciably again at considerably higher concentrations (46 5 and 110 8 mg per cent) in 2 exp does not carry much weight since the determinations of the conversion rate at such high concentrations is quite inaccurate (low extraction fractions rate of urinary excretion approaching the rate of renal extraction) In spite of this inaccuracy it seems safe to conclude that the renal rate of glycerol conversion does not rise at arterial blood concentrations above approximately 20 mg per cent and that it may decline at higher levels Enzyme inhibition by excess substrate or by conversion products might be the cause of a decline It apparent then that at arterial blood concentrations up to roughly 50 mg cent the rate of conversion of glycerol in the kidneys exceeds the rate of absorption (cf Fig 3) whereas at very high concentrations the rate of reabsorption will exceed the rate of conversion

At the saturation level (arterial blood concentration of 15—30 mg per cent) where determinations could be made with satisfactory accuracy the rate of renal conversion of glycerol was found to be on an average 0 087 mg per minute per gram of kidney or 0 88 mg per minute per kg of body weight The rate of conversion of glycerol in the kidneys amounted to about 1/5 (range 13 7 to 29 per cent) of the rate of conversion in the body as a whole (calculated as the rate of infusion of glycerol at constant blood concentration minus the rate of urinary excretion) The observed whole body conversion rate (at saturation) is in good agreement with that reported by HOLST (1944)

A few stop-flow experiments were made (during mannitol diuresis in cats) in an attempt to localize the site(s) of glycerol reabsorption At high blood concentration levels (80—95 mg per cent) the glycerol concentration in the first effluents was significantly higher than the concentration of the free flow urine and it fell off to approximately the level of the latter in the subsequent effluents Rather similar patterns were observed for urea and inulin In an experiment made at an arterial plasma glycerol concentration of 22 mg per

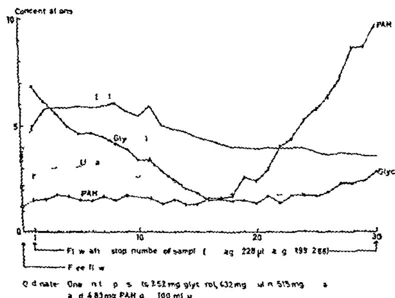


Fig 4 *Stop flow experiment* Upon unclamping the ureter approximately 300 µl of urine was allowed to spill before collection of sample 1 was started
The PAH concentration in samples 0—18 originated from a dose administered in a stop-flow experiment prior to the one illustrated The rise in concentration appearing from sample 19 and onwards is caused by a PAH dose injected intravenously about 45 seconds before unclamping the ureter
Plasma glycerol concentration during the stop ?? mg per cent

cent (cf Fig 4) a rise in the glycerol concentration in the first effluents above the free flow level was also observed but in this case the glycerol concentration fell off steeply in the subsequent effluents to reach a minimum of 40 per cent of the free flow level nearly coinciding with the appearance of PAH (injected about 45 sec before terminating the stop)

Since any glycerophosphate present in the samples would be included in the determinations of glycerol by the enzymatic method a conversion of glycerol to glycerophosphate and the subsequent appearance of the latter in the renal venous blood would make the calculated renal conversion rates too low Excretion of glycerophosphate in the urine would have the same effect and furthermore it would make the calculated reabsorption rates too low Errors of this kind are however hardly of any importance since in one experiment where glycerophosphate was determined (by leaving out glycerokinase) no arterio renal venous difference and no urinary excretion of glycerophosphate could be demonstrated The arterial and renal venous) blood concentration of this substance did however rise slowly to about 1 mg per cent expressed as glycerol) during the experiment with glycerol concentrations in arterial blood rising to about 60 mg per cent)

Discussion

The general pattern of the renal excretion (reabsorption) of glycerol observed in this study is in agreement with that observed by SVEINSSON (1948) in studies on normal human subjects (using a less specific method for glycerol analysis and omitting determination of the filtration rate)

The fact that with increasing filtered glycerol loads the rate of glycerol reabsorption continues to rise while the reabsorption fraction appears to converge upon a limiting value is readily explained on the assumption that at high filtered loads the reabsorption is predominantly due to back diffusion The rather small size (about 0 4) of the reabsorption fraction (at high loads) in comparison with that of water (about 0 9—0 95) indicates that the permeability to glycerol must be rather low in the cells of the distal tubular system

The fact that at low filtered loads the glycerol concentration of the urine may become lower than that of the arterial blood plasma (2 63 mg per cent and 7 1 mg per cent respectively in a particular case) would traditionally be interpreted as due to active reabsorption It may however well be explained without resorting to a particular active transcellular transfer mechanism for glycerol Since at low filtered loads the rate of renal conversion of glycerol is considerably higher than the rate of reabsorption all glycerol reabsorbed at low filtered loads might be converted to some other product(s) in the tubular cells It seems reasonable to assume that at the lowest glycerol concentrations in arterial blood plasma studied here (5—8 mg per cent) the absorption of glycerol is exclusively effected by a diffusion into the tubular ls followed by — or rather caused by — an intracellular conversion into her products

The uptake and conversion of glycerol in the tubular cells is however not limited to that presented to them from the luminal side (the filtered load) as indicated by the fact that the rate of conversion exceeds the rate of reabsorption at low filtered loads Thus the tubular cells — or at least some of them — are able to take up glycerol from the basal side too and it is reasonable to assume that this uptake also occurs by diffusion

This concept of the handling of glycerol at low filtered loads — uptake of glycerol in some tubular cells by diffusion through the luminal as well as the basal cell membranes as a consequence of an intracellular conversion — is not incompatible with the observation that at low glycerol concentrations of the arterial plasma the glycerol concentration of the urine may become somewhat lower than the concentration in the mixed renal venous blood and that the extraction fractions at such loads do not become higher than about 0 5—0 6 Such findings could be obtained if the permeability to glycerol of the glycerol converting cells were lower at the basal than at the luminal surface, it would even suffice if such permeability characteristics were present only in some glycerol-converting distal cells In interpreting the above mentioned observa

tions it may also be recalled that the measurements were made on mixed renal venous blood the glycerol concentration in some parts of the venous blood (and some parts of the peritubular interstices) may have been appreciably lower than that of the mixed venous blood It is possible that the extraction fraction is appreciably higher at arterial glycerol concentrations below 5 mg *per cent* but *it was deemed impossible to measure* the extraction fraction in this range with acceptable accuracy with the present analytical method [1]

The site of the metabolic conversion (and conversion reabsorption) of glycerol in the tubules is not known at present The results of the stop flow experiment shown in Fig 4 confirm that the permeability to glycerol must be low in the most distal parts of the nephron and show that glycerol may be reabsorbed in proximal parts of the nephron to the extent that the concentration in the tubular fluid becomes appreciably lower than that of the arterial plasma The findings do not exclude that some reabsorption of glycerol (and metabolic conversion) may take place at a distal site e g in the convoluted tubule of II order

Altogether our observations may be accounted for on the basis of a set of assumptions like the following Glycerol is converted in the proximal tubular cells and in some cells in the distal tubular system (convoluted tubules of II order?) into other products (CO glucose lactate?) The former cells are assumed to be readily permeable to glycerol at both the luminal and to the basal surface whereas the latter are assumed to be permeable only at the luminal surface The collecting tubules are assumed to be practically impermeable to glycerol At very low filtered loads glycerol is reabsorbed almost completely by conversion reabsorption but the major part of the glycerol converted in the proximal cells is taken up from the peritubular blood As the filtered load rises the glycerol conversion mechanism approaches saturation and as the concentration in the proximal tubular fluid increases in consequence a proportionally greater part of the glycerol converted must be assumed to gain access to the cell from the luminal side the rate of conversion reabsorption consequently rises somewhat At the same time the elevation of the glycerol concentration in the proximal tubular fluid will cause a transcellular diffusion of glycerol through the proximal tubular wall and with further rises in the filtered load the reabsorption by transcellular back diffusion becomes quite dominant in comparison with the conversion reabsorption which is incapable of any further increase (or which may even fall due to enzyme inhibition)

[1] To interprete the fact that glycerol extraction fractions higher than about 0 5—0 6 were not observed the possibility may also be considered that some glycerol might diffuse from the beginning of the peritubular capillaries into the blood leaving the cortical interstices. An evaluation of the magnitude of such a counter-current exchange is hardly possible from our present knowledge of the arrangement of the small blood vessels of the renal cortex A similar shunting might contribute to the incompleteness of extraction of PAH and Diodrast at even very low plasma concentrations.

The first step in the metabolic conversion of glycerol in the kidney is un questionably the formation of glycerophosphate by means of glycerokinase It is noteworthy that our in vivo findings of a renal rate of glycerol conversion of about 20 per cent of that of the body as a whole (at the saturation level — about 15—20 mg per cent — apparently nearly the same for liver and kidney) are in close agreement with WIELAND s (1957 b) observations on the distribu tion of glycerokinase He found almost identical concentrations of this enzyme in liver and kidney (from rats) and absence of the enzyme in a number of other tissues The liver/kidney weight ratio is close to 4 1

The present study does not disclose the further fate of glycerol in the kidneys Besides complete oxydation a conversion into glucose (or lactate) is likely to occur In a single experiment where the arteriovenous oxygen difference was determined the rate of oxygen consumption in the kidneys (3 15 ml/min) was found to be somewhat lower than that (3 66 ml/min) required for complete oxidation of the glycerol converted The infusion of glycerol was found to be accompanied by a drop *in the RQ from 0 91 to 0 81* which may have been due to a partial oxydation of glycerol (e g conversion into glucose) The arteriovenous glucose difference expectable from a partial conversion of the glycerol metabolized into glucose was deemed to be too small for detection

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From the Department of Pharmacology University of Goteborg and the Department of Histology Karolinska Institutet Stockholm Sweden

Evidence of Dopamine Containing Neurons in the Retina of Rabbits

By

J HAGGENDAL and T MALMFORS

Recently adrenergic neurons have been demonstrated in the retina of rats with a fluorescence microscopical technique (MALMFORS 1963) The neurons were situated in the inner part of the inner nuclear layer and had their synaptic terminals in a well defined zone in the outer part of the inner plexiform layer It was suggested that they contained noradrenaline or dopamine The same type of cells with essentially the same distribution was found in the retina of rabbits (MALMFORS unpublished results) This investigation was performed in order to study the identity of the catechol amine found in the retina

The experiments were performed on rabbits one on retinas from 4 animals and another on retinas and choroids from 13 both albino and pigmented rabbits The animals were killed by air embolism The eyes were immediately dissected out and halved 2 mm behind the limbus The retina was then prepared free from the choroid and cut from the optic papilla in order to exclude catechol amines in the vascular nerves there (MALMFORS unpublished results) In the second experiment the retina was prepared as described above and the choroid was prepared free from the sclera The material was immediately put in ice cold 0 4 N perchloric acid and homogenized according to BERTLER CARLSSON and ROSENGREN 1958 The pH of the extract was adjusted and passed through a strong ion exchange column Dowex 50 –X8 After washing with water and buffer the elution was performed with N hydrochloric acid in 1 ml fractions (for details of the column procedure see HAGGENDAL 1963) The fluorescence obtained by treating every 1 ml fraction according to the trihydroxyindole method was read at the activating and fluorescence peaks of noradrenaline respective dopamine Noradrenaline was estimated according to HAGGENDAL 1963 dopamine according to CARLSSON and WALDECK 1958 modified by CARLSSON and LINDQVIST 1962

Two peaks were found in the determination of the retina The peaks were chromatographically typical for noradrenaline and dopamine (cf BERTLER CARLSSON and ROSENGREN 1958 HAGGENDAL 1962) The noradrenaline peak fraction 2 was very weak The dopamine peak was evident with maximum in fractions 6 to 8 and these fractions had activating (330 $m\mu$) and fluorescence

Table I Catechol amines in ng per retina and choroid respectively

	Noradrenaline	Dopamine	No of rabbits
Retina	0 5	13	4
	0 05	8	13
Choroid	10	0	13

(380 mμ) peaks typical for dopamine The corresponding estimation of the choroid showed a rather high noradrenaline peak but no evident dopamine peak The amounts are given in Table I

These results show that dopamine is the dominating catechol amine in the retina of rabbits The noradrenaline found in the retina may be a contamination from the choroid The dopamine found in the retina is probably localized to the adrenergic neurons demonstrated there with the fluorescence microscopical technique The presence of dopamine in these cells fits well with the findings of MALMFORS (unpublished results) that there is no visible reduction histochemically of the catechol amine fluorescence in the retina of rats 24 hours after two injections of 400 mg/kg body weight α methyl metatyrosine with 3 hours interval From biochemical experiments it is known that such treatment lowers the noradrenaline level while the dopamine level in the brain is only slightly influenced (HESS, OZAKI and UDENFRIEND 1960 BRODIE MAICKEL and WESTERMAN 1961 CARLSSON and LINDQVIST 1962)

This work has been supported by a research grant (NB 07834 03) from the United States Public Health Service by Svenska Sällskapet för Medicinsk Forskning and by the United States Air Force under Grant No AF EOAR-63 14 and monitored by the European Office Office of Aerospace Research

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Acta physiol scand 1963 59 297—298

From the Department of Physiology Karolinska Institutet Stockholm Sweden

Recording of Net Ionic Flux during a Single Action Potential in Nitellopsis Obtusa

By

L HAAPANEN and C R SKOGLUND

On the basis of isotope studies the ionic changes occurring in various types of excitable cells after periods of repetitive stimulation have been analyzed and suitable calculations made to determine the ionic flux in the course of a single activation Attempts have also been made to determine the temporal relationship between the ionic fluxes and the electric events by fractionating the tracer outflow from certain slowly reacting cells SPYROPOULOS TASAKI and HAYWARD (1961) have thus established the average time course of the potassium efflux during activation in *Nitella* by collecting portions of the fluid containing the the radioisotope efflux at 0 75 sec intervals during 40 successive cycles of activity The aim of the present investigation has been to obtain a method of recording directly the net ionic flow in *Nitellopsis obtusa* coincident with a single action potential

Single internodal cells of the algae were placed in a four chambered perspex container soft paraffin seals providing insulation between the chambers (Fig 1) A capacitance meter connected to a capacitance probe placed in the extra cellular fluid was used to measure the changes in ion flux during the course of a single action potential and these changes were displayed on a two-channel inkwriter simultaneously with the action potential This meter described previously (HAAPANEN 1962) gives an output of 11 V for a capacitance change of 0 001 pF Titration methods making use of the principle of capacitance measurements have been described by *e g* BLAEDEL and MALMSTADT (1950) The two types of probes used were (i) a completely insulated U shaped silver tube of 2 mm length and an inner diameter somewhat larger than that of the internodal cell the inside insulation consisting of a plastic sheet about 15 μ thick and (ii) a Ag AgCl electrode connected to the capacitance meter through a capacitor of a few pF The same electrode could be used as one lead in the action potential recording

Based on a larg number of measurements the following characteristics of these capacitance probes in solutions of different concentrat ons can be described At low ionic concentrations small increments in solute result in a decrease in total capacitance of the probe with intermediate concentrations the sensitivity to increments in solute reaches a maximum and the capacitance changes become positive while at high concentrations the capacitance changes remain positive but sensitivity is reduced At the working frequency of the meter (1 085 kc/sec) maximum sensitivity was attained in a solution whose conductivity was about $10^{-4}ohm^{-1}cm^{-1}$ (*e g* 1 mM KCl) At this concentration an increment of conductivity of 0 1 could easily be detected In this series of experi

Table I Catechol amines in ng per retina and choroid respectively

	Noradrenaline	Dopamine	No of rabbits
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This work has been supported by a research grant (NB 02854 03) from the United States Public th Service by Svenska Sallskapet for Medicinsk Forskning and by the United States Air e under Grant No AF EOAR 63 14 and monitored by the European Office Office of Aerospace Research

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Acta physiol scand. 1963 59 299—318

From the Department of Physiology Kungl Veterinarhogskolan Stockholm 50 and the Gustaf Werner Institute for Nuclear Chemistry Uppsala Sweden

Radiation Induced "Hypophysectomy" and Hypothalamic Lesions in Lactating Goats

By

CHARLES C GALE[1] and BORJE LARSSON

Received 25 February 1963

Abstract

GALE C C and B LARSSON *Radiation induced hypophysectomy and hypothalamic lesions in lactating goats* Acta physiol scand. 1963 *59* 299—318 — Radiolesions were produced in the pituitary gland and/or median eminence pituitary stalk region of seven lactating goats by use of a narrow collimated beam of 185 MeV protons from a synchrocyclotron Radiation doses of 25 to 30 krad were restricted to these structures by rotation of the goats heads around the vertical and horizontal axes of the planned lesion Ten to 15 days after pituitary irradiation in four goats milk production declined steeply over a ten day period to about 20 per cent pre lesion levels In two of the hypophysectomized animals with injury also in the median eminence diabetes insipidus developed concurrently with the onset of lactation block Daily replacement with prolactin (luteotrophin) somatotrophin ACTH triiodothyronine and insulin had restored milk synthesis to 60 per cent of pre radiation levels when destruction of trigeminal nerve trunks by continued lateral spread of radiolesions caused an abrupt decline in the health of the goats

In 3 lactating goats irradiation of the median eminence/pituitary stalk region was followed after 25 to 30 days by (1) disappearance of the milk ejection reflex (2) onset of diabetes insipidus characterized by absence of the normal interphase (3) decline in milk production to 30 to 40 per cent of pre lesion levels and (4) in one of them marked loss of hair That median eminence lesions blocked lactation secondary to pituitary insufficiency of ACTH TSH and STH but not of prolactin or of oxytocin was suggested by the ability of varied therapeutic regimens to restore lactation

[1] Postdoctoral Research Fellow Division of Neurological Diseases and Blindness U.S Public Health Service

to fail to block prolactin secretion in progress e g, pituitary stalk section (JACOBSOHN 1949), transplantation of the adenohypophysis away from the median eminence (EVERETT 1956 a) median eminence lesions (McCANN and FRIEDMAN 1960 HALÁSZ and SAWYER 1960 GALE *et al* 1961) and *in vitro* incubation of the adenohypophysis (NICOLL and MEITES 1962)

Since previous studies of central control of prolactin secretion and milk production have been performed primarily in small laboratory animals it was thought important to investigate the role of the CNS in pituitary gland regulation during lactation in a ruminant — in the present study the goat COWIE and TINDAL (1961) have shown that surgical removal of the hypophysial lobes causes lactation to stop in this species, and that replacement with prolactin somatotrophin (STH), ACTH or adrenal corticoids triiodothyronine and insulin affords marked restoration of milk production That injury to the median eminence/pituitary stalk region may block milk synthesis in the goat has been reported by TVERSKOI (1960) but it was not determined which hormonal deficits caused this impairment In the present study lactational performance was studied in goats subjected to proton irradiation designed either to destroy the hypophysis or to interrupt the hypothalamic/hypophysial connections in the median eminence By this technique which was suggested by TOBIAS and co workers lesions can be produced in the CNS without causing significant trauma or stress to the animals (cf LEKSELL *et al* 1960) Following the onset of lactation block replacement therapy was given to identify hormonal deficiencies

Since median eminence lesions cause an insufficiency in secretion of neurohypophysial hormones secondary to interruption of the supraopticohypophysial tracts (FISCHER INGRAM and RANSON 1935) and since diabetes insipidus has not previously been studied in the goat water metabolism was also investigated in these animals

Methods

1 *Study of lactation* Daily milk production in nine horned goats was measured 2—4 weeks before irradiation and then continuously during the entire experimental period Milking was performed twice daily at about 0930 and 1700 hours with particular care being taken to empty the udder completely by massage The lactation studies were begun in early spring after parturition when milk production was highest but some animals in the series were irradiated in early autumn (Table 1) Owing to the slow development of radiolesions and to the chronic nature of the study investigation in certain animal continued therefore into winter Since milk production in goats may decline in winter — largely due to the advancing state of a new pregnancy a complication avoided in this study — the immediate pre lesion lactation level was used as the base of reference for each animal

Milk ejection reflex The milk ejection reflex was tested as follows the milk from one udder half was initially drained by insertion of a small metal cannula into the teat During the rapid insertion of the cannula (avoiding undue stimulation of the teat) the goat was distracted by the procedure of gripping its horns and gently shaking its head

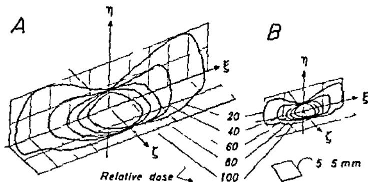


Fig 1 *Typical distribution of absorbed dose in goats irradiated for (A) total hypophysectomy and (B) destruction of the median eminence (see also Table 1)* The center of rotation (= origo) was located approximately in the middle of the target structures the η plane being in the median plane of the skull with the ζ axis approximately parallel with the base of the skull Isodose curves are shown for $\eta = 0$, > 0 and $\zeta = 0$ $\eta > 0$ The complete distribution of dos may be visualized by reflection in the $\xi\eta$ plane and the $\zeta\xi$ plane respectively

In this manner reflex release of oxytocin was avoided After permitting the animal to rest for five to ten minutes with the cannula *in situ* the udder on the opposite side was milked in the usual manner A positive milk ejection reflex was signified by an abrupt streaming forth of 20 to 30 ml of milk from the cannula 30 to 50 sec after the beginning of milking Absence of this gush of milk was considered a negative milk ejection reflex In negative cases an *i v* injection of 30 mU of oxytocin (Syntocinon Sandoz) was administered to demonstrate that the mammary alveoli had contained sufficient amounts of milk to indicate endogenous oxytocin secretion

Other measurements Occasionally specific gravity of milk cooled to room temperature was determined Concentration of Na^+ and of K^+ in milk and urine samples were measured on an EEL flame photometer and Cl^- was measured by the method of BRUN (1949)

2 *Replacement therapy* Following the decline in milk production after central lesions various combinations of hormones were administered subcutaneously in the neck region of the animals in an attempt to restore lactation Daily doses were given of prolactin (luteotrophin LTH) NIH P S 3 Ovine[1] 300 I U somatotrophin (STH growth hormone) NIH GH S 4 Ovine[1] 12 mg ACTH Schering Corporation ACTH Depot 5 I U triiodothyronine (T3) Smith Kline and French[2] 1 0 mg and protomine zinc insulin Vitrum 8 I U

3 *Technique for irradiation* The radiological characteristics of the 185 MeV proton beam have been described elsewhere (cf LARSSON 1961 and 1962) and so have the dosimetry and other details of the radiosurgical procedure (LEKSELL *et al* 1960 ÅKERMAN *et al* 1962) For the present experiments a special head holder was designed to clamp onto the horns of the goats so as to permit rigid fixation of the skull or irradiation under slight sedation with chlorpromazine Continuous rotation of the head was applied during irradiation so as to destroy the pituitary gland/ or the median eminence/ pituitary stalk region more or less without affecting surrounding nerve tissues In this way doses of 25—30 krad were absorbed in the tissue at the center of the target volume Complete calculated dose distributions are shown in Fig 1 In some goats the radiation

[1] The prolactin and the somatrophin were gifts of the Endocrine Study Section National Institutes of Health U S P H S

[2] The triiodothyronine was a gift of Smith Kline and French Co

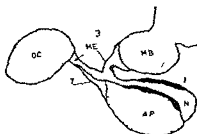


Fig 2 *Diagram of the hypophysis and ventral hypothalamus of the goat (median sagittal plane)* Destruction of the hypophysial lobes *per se* (AP I N) by radiolesions termed simple hypophysectomy caused a marked decline in milk production. When radiolesions were produced in the median eminence/pituitary stalk region as well as in the hypophysial lobes termed total hypophysectomy development of diabetes insipidus occurred concurrently with the block in lactation. Production of radiolesions in the median eminence alone caused diabetes insipidus disappearance of the milk ejection reflex and a less severe impairment in lactation

OC	optic chiasm
ME	median eminence (arrows indicate the approximate rostral and caudal extent)
MB	mammillary body
3	third ventricle
AP	anterior lobe of the pituitary gland
N	neural lobe of the pituitary gland
I	intermediate lobe of the pituitary gland
T	pars tuberalis

procedure had to be repeated before appreciable physiological changes appeared. Numerical values of significant physical parameters defining the radiation procedure in each animal are given in Table I

4 *Routine care of the animals* All goats were maintained in metabolism cages except on the day of irradiation when they had to stay at the cyclotron laboratory. They were given daily a ration of 250 g of grain with ½ g of NaCl added. Water and hay were available ad libitum. Daily measurements were made of water intake, urine volume and specific gravity and rectal temperature. Average daily room temperature ranged from a low in winter of 17 C to a high of 22 C in summer

5 *Histology* The animals were killed by decapitation under Nembutal anesthesia. Perfusion through the carotids was rapidly performed at 150 mm Hg with physiologic saline followed by 5 % formol saline both solutions being at room temperature. The brain was removed with the pituitary gland attached and fixed for about one week in 5 formol saline. A block of tissue containing the diencephalon was dissected out embedded in celloidin and cut in transverse serial sections at 35 μ. Alternate sections were stained with toluidine blue and by the method of LOYEZ (cf CULLING 1957). Usually the pituitary gland was dissected free from the brain following fixation embedded in paraffin cut serially at 5 to 15 μ and stained by LADEWIG's (1938) modification of MALLORY's method. The ovaries and the uterus were dissected out, weighed and representative samples were fixed in 5 formol saline embedded in paraffin cut at 15 μ and stained with hematoxylin and eosin

Results

I "Hypophysectomy" produced by proton irradiation

A *Block of lactation after irradiation of the hypophysis*

In order to investigate the importance of the pituitary gland in lactation in the goat four lactating animals were subjected to proton irradiation designed to destroy the hypophysis. In two of these animals "Lillie" and "Enspent" destruction of the pituitary lobes *per se* was attempted — a procedure which

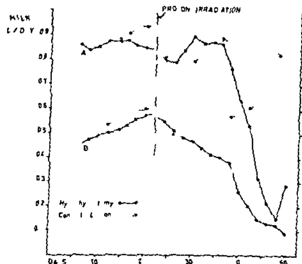


Fig 3 Block in lactation following radiation induced hypophysectomy Marked decline in milk production resulted in two goats subjected to simple hypophysectomy induced by irradiation of the hypophysial lobes Both animals remained in apparent good health and nutritional balance during the period illustrated Note that irradiation caused very little immediate impairment in lactation indicating that the goats experienced very little stress during the experimental procedure Control animals received non destructive doses of protons in the median eminence region

may be described as producing simple hypophysectomy In two other goats "Floyd and '100 the proton beam was so directed as to ablate in addition to the pituitary lobes the median eminence/pituitary stalk region i e the portion of the neurohypophysis projecting up into the hypothalamus and forming the floor of the third ventricle (Fig 2) This latter radiolesion may be described as causing total hypophysectomy

Ten to 15 days after effective irradiation which acutely caused only very slight transient decline in lactation there was observed in all 4 goats a marked drop in milk production After a period of about 10 additional days the milk yield had fallen to approximately 20 % of pre radiation levels Two control animals White and "Caroline which had been irradiated with non destructive dosages in the median eminence did not show lactational impairment (Fig 3 Table I) One goat subjected to simple hypophysectomy (Enspent) displayed a spontaneous recovery in lactation to about 40 per cent of pre lesion levels Subsequent histology showed that it had incurred about 60 to 70 % destruction of the adenohypophysis The other three animals did not exhibit spontaneous recovery and later their pituitary glands were found to be totally or near totally destroyed The ability of radiation hypophysectomy to impair milk synthesis so markedly in goats which remained in good condition and continued to eat their daily food ration demonstrates that the hypophysis is essential for lactation in this species

In the two animals, Floyd and 100 which were totally hypophysectomized the reduction in milk yield was accompanied by the onset of diabetes insipidus (Fig 4) No marked increase in water turnover however was seen in the 2 goats Lille and Enspent in which only the hypophysial lobes had been irradiated

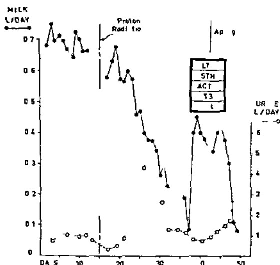


Fig 4 *Restoration of lact tion by re placement therapy in Floyd a totally hypophys ctomi ed goat* Decline in milk production following irradia tion of the pituitary gland and me dian eminence was associated with the development of diabetes ins pidus which abated as milk synthe sis reached very low levels Replace ment daily with prolactin (LTH) (300 I U) STH (12 mg) ACTH (5 I U) T3 (1 mg) and insulin (8 I U) quickly restored milk pro duct on to 60 per cent of pre lesion levels The antidiuretic activity of the prolact n p eparation is bel eved to have prevented an augmentation in water turnover by replacement with ACTH and T3 Failure to eat secondary to trigeminal nerve dam ag caused a rap d decline in the health of this animal

B Restoration of lactation by hormonal replacement

When lactation had declined to very low levels in the 4 hypophysectomized goats replacement therapy with various combinations of hormones was initiated in the attempt to restore lactation and to identify the constellation of pituitary hormones essential for milk synthesis Administration daily of prolactin (LTH) ACTH STH triodothyronine (T3) and insulin for periods of 5—10 days afforded restoration of milk production to 60 per cent of pre radiation levels (Fig 4 Table I) At that time however it became apparent that the radio lesions after destroying the pituitary lobes had continued to expand laterally and to injure gradually the trunks of the trigeminal nerve lying adjacent to the pituitary Damage to the mandibular branch of the tregeminus indicated by atrophy of the masseter (chewing) muscles then prevented these animals from eating their normal diet of hay and grain Due to the difficulty of maintaining force fed goats in good condition the health of these animals deteriorated rapidly and they were killed for histological study

C Histology of hypophysectomized goats

Microscopic examination of the pituitaries showed total to near total destruction of the hypophysial lobes in 3 (Lilie Floyd and 100) of 4 goats (Fig 5) the fourth animal (Enspent) showing 60 to 70 per cent destruction of the adenohypophysis Two animals in which total hypo physectomy was attempted (Floyd and 100) exhibited, in addition destruction of the median eminence/pituitary stalk region (Fig 6) Trigeminal nerve injury was present in all 4 goats and was marked by demyelinization of nerve trunks and round cell infiltration



Fig 5 Photomicrograph of hypophysis destroyed by proton irradiation in goat Lloyd

A The pituitary gland (transverse section) shows total destruction of the adenohypophysis (A) and near total destruction of the neurohypophysis (N an I I) Stain Ladewig

B Magnification of an area of adenohypophysis (shown above) destroyed by proton irradiation Note loss of cellular detail

C Magnification (the same as in B) of an area of adenohypophysis from a normal goat Stain Ladewig

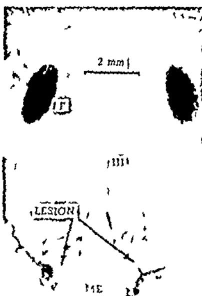


Fig 6 Destruction of the median eminence by proton irradiation in goat Floyd Bilateral destruction of the medium eminence at the base of the hypothalamus (transverse section) is shown Note that there is an absence of tissue reaction at the junction of the radiolesion with viable parenchyma This lesion produced diabetes insipidus in this animal F Fornix, ME median eminence III third ventricle Stain Loyez

II Effect of radiolesions in the hypothalamus

A Block of lactation after production of radiolesions in the median eminence

Following the study of hypophysectomy in goats, which served to confirm the essential role played by the pituitary gland in lactation, an investigation was made of the importance of the central nervous system in regulating hypo-

Fig 7 A. Effect of a median eminence radiolesion on lactation in goat "Esmeralda" Radiation of the median eminence caused a decline in milk production which was associated with the loss of the milk ejection reflex and the development of diabetes insipidus (see Fig 7 B) Very marked hair loss from the neck and back also occurred in this animal (Fig 9) Administration of LTH for seven days failed to improve the depressed lactation, but when ACTH, T3, STH and insulin were given, milk production was rapidly restored. Addition of LTH to this regimen failed to improve lactation further The onset of aphagia shortly thereafter caused a quick deterioration of the health of the animal

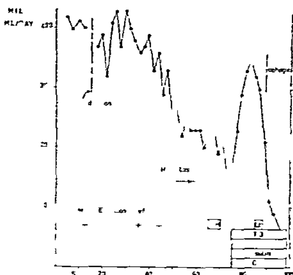
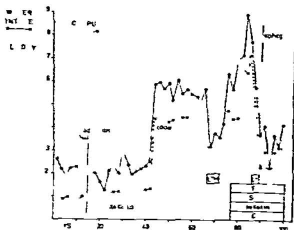


Fig 7 B Production by median eminence radiolesions of diabetes insipidus lacking the normal interphase Thirty days after irradiation of the median eminence "Esmeralda" exhibited an abrupt onset of diabetes insipidus. This elevation of water turnover was unusual in that it lacked the normal interphase of diabetes insipidus, i.e., the brief period, following the initial large increase in water turnover when water metabolism returns to normal. Rather it appears that permanent phase of diabetes insipidus came on directly When LTH was given an amelioration of the elevated water turnover occurred. Administration of ACTH STH T3 and insulin later increased water turnover sharply but failure of the animal to eat caused an abrupt reduction in the severity of diabetes insipidus.



physial function during lactation An attempt was made in 3 lactating goats to interrupt the connections between the hypothalamus and the pituitary gland by producing radiolesions in the median eminence pituitary stalk region but without injuring the hypophysial lobes *per se* From the previous experience with "hypophysectomized goats which developed lesions in the trigeminal trunks the absorbed dose was reduced in order to restrict tissue damage to the desired structures However either the dose elected or the area irradiated was

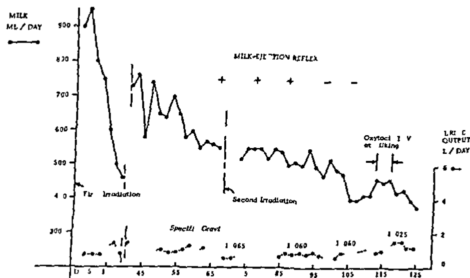


Fig 8 A Effect of median eminence radiolesions in goat In o After the first proton radiation milk production declined briefly but was restored spontaneously — the milk ejection reflex remained positive Following a second radiation of the median eminence however a gradual decline in lactation associated with the loss of the milk ejection reflex occurred Water turnover was not elevated but specific gravity of the urine was lowered That this oxytocin deficiency did not prevent complete milk removal was indicated by the failure of post milking injections of oxytocin to permit the removal of significantly greater quantities of milk upon renewed milking

apparently too small to produce significant brain damage since the animals failed to show signs characteristic of lesion development during the several months of careful post irradiation study Two of these goats Esmeralda and Ingo were therefore given a second proton dose while in the case of Snowflake it was found necessary to administer a third proton treatment (Table 1) Twenty to 35 days after these additional proton exposures the animals exhibited a decline in milk production which eventually fell to about 35 % of pre lesion levels (Fig 7a 8a and 10) The start of this decline was marked by the onset of mild to severe diabetes insipidus and by the disappearance of the milk ejection reflex indicating deficiencies of posterior lobe hormones When oxytocin was injected i v immediately after the twice daily milkings in Ingo for a 7 day interval the additional amount of milk which could be obtained by further milking was very small 10 to 15 ml (Fig 8a) This evidence that oxytocin deficiency did not appreciably prevent milk removal in these animals is in agreement with findings of other workers (TVERSKOI 1960) Of special interest was the absence of the normal interphase in the diabetes insipidus shown by these animals (Fig 7b), an effect that was also noted in the two totally hypophysectomized goats earlier A further unexpected finding was the marked loss of body hair which occurred in Esmeralda (Fig 9) another animal Snowflake showed moderate hair loss

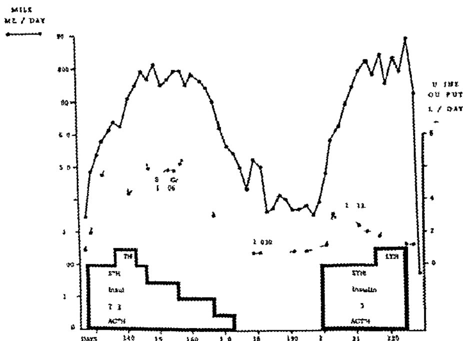


Fig 8B *Restoration of lactation in goat In... by replacement therapy* When milk production had declined to 45 per cent of pre-radiation level (175 days after the first proton exposure) replacement daily with ACTH T3 insulin and STH evoked a prompt restoration of milk synthesis and also a large increase in water turnover The brief administration of LTH also during the rising phase of restoration of lactation apparently did not contribute to this improvement since a second restoration to the same high level was achieved by administration of replacement excluding LTH and since addition of LTH to the therapeutic regimen failed to further increase the high level of milk production so attained Selective withdrawal of hormones from the regimen following lactational restoration suggests that ACTH T3 insulin and to a lesser extent, STH were important in stimulating milk synthesis Very rapid decline in lactation after cessation of the second therapeutic treatment was associated with development of motor impairment secondary to radiolesions in the cerebral peduncles (Fig 11)

B *Restoration of lactation by hormonal replacement*

In two of three goats (Esmeralda and Ingo) with lactation blocked by median eminence lesions the daily administration of ACTH STH triiodothyronine and insulin — but not of prolactin — restored milk production to or near pre lesion levels (Fig 7a and 8b Table I) In Esmeralda the giving of prolactin alone during the period of depressed milk synthesis failed to increase milk production while the adding of prolactin to the therapy in Ingo could not be shown to improve lactation further Selective withdrawal of hormones from the the regimen following lactational restoration in Ingo suggests that ACTH T3 insulin and to a lesser extent STH were important in stimulating milk synthesis Fig 8b) After lactation had been restored in "Esmeralda by therapy excluding prolactin the development of aphagia and



Fig 9 *Loss of hair from the neck and back of goat Esmeralda after median eminence radiolesions* About 35 days after median eminence irradiation there was observed a marked loss of hair which denuded the neck and back region As shown whole hand fulls of hair could easily be pulled out This hair loss occurred after the decline in lactation loss of the milk ejection reflex and onset of diabetes insipidus (Fig 7 A and 7 B)

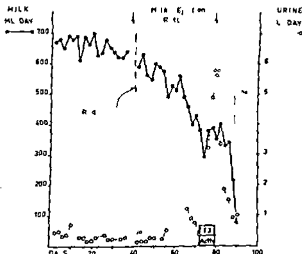


Fig 10 *Effect of median eminence radiolesions in goat Snowflake* After a third exposure to proton irradiation this goat displayed a decline in milk production loss of the milk ejection reflex and onset of diabetes insipidus An abatement of the elevated water turnover paralleled the fall in milk synthesis Brief treatment with ACTH and T3 failed to restore milk production — at best merely checked the downward trend — but produced a marked increase in water turnover Subsequent development of aphagia and partial blindness was related to the progressive development of radiolesions lateral to the diencephalon and in the optic tracts (Fig 13 A)

the abrupt deterioration in the animal s health due to untoward brain damage precluded an evaluation of the effect of selective hormonal withdrawal In the third animal Snowflake replacement with ACTH and T3 failed to restore milk production appreciably although the severity of diabetes insipidus was augmented greatly (Fig 10) The general health of this animal also declined following signs of unfavourable extension of brain lesions e g aphagia partial blindness

C *Signs of damage of other parts of the brain than the median eminence*

In all 3 goats with median eminence lesions the onset of diabetes insipidus and/or the loss of the milk ejection reflex served to measure the time after



Fig 11 *Postural impairment after development of radiolesions in the cerebral peduncles and internal capsule* 120 days after the second proton exposure in Ingo this goat abruptly developed a gross postural disturbance characterized by a bulldog like stance and wing scapula Despite this marked impairment alimentation was not affected Histology revealed the presence of radiolesions in the cerebral peduncles and internal capsule (Fig 13 B) in addition to the median eminence

proton irradiation for the gradually developing radiolesions to destroy the supraoptico-hypophysial tracts and to block secretion of posterior pituitary hormones After the 3 animals had manifested this sign of median eminence injury, they remained in good health for periods of 5 weeks(Snowflake) 7 weeks (Esmeralda) and 22 weeks (Ingo) It then became evident that due to the repeating of the proton treatments radiolesions developed in other portions of the brain In the case of Snowflake and Esmeralda the failure to eat despite a retained ability to chew and swallow food placed in the mouth resulted in a rapid decline in health In addition both animals became partially blind before they were killed for histology Ingo developed a gross motor disturbance characterized by a bulldog liked stance and wing scapula 22 weeks after loss of the milk ejection reflex (Fig 11) Although this animal experienced difficulty in standing and walking it continued to eat and drink normally and had no apparent visaul impairment when killed several days later

These neurological symptoms were most likely due to damage to portions of the brain lying lateral to the median eminence (see below) and seemingly resulted from the overlapping of sub lethal proton doses consequent to the repeated exposures It is likely that these lateral lesions rather than those along the midline caused the abrupt deterioration in health of these animals Lesions in the far lateral hypothalamus and in the globus pallidus have been reported to produce aphagia in other species (ANAND and BROBECK 1952 MORGANE 1962) Further it is unlikely that the lateral injury influenced significantly the lactational performances studied earlier in these animals since the lateral lesions should have developed more slowly than those near the midline in the region of maximal dose

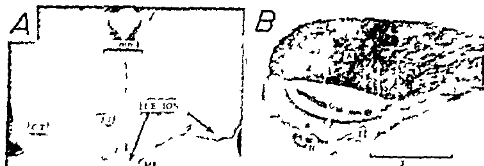


Fig 12 Photomicrographs of median eminence radiolesion and the effect on the hypophysis in goat Esmeralda

A Bilateral destruction of the median eminence in the basal hypothalamus (transverse section) is illustrated ME = median eminence III = third ventricle Stain toluidine blue

B Photomicrograph of the hypophysis (midline sagittal plane) of the same animal shows atrophy of the neural lobe (N) little obvious change in the intermediate lobe (I) and absence of marked central necrosis in the anterior lobe (A) C colloid cyst Stain Ladewig

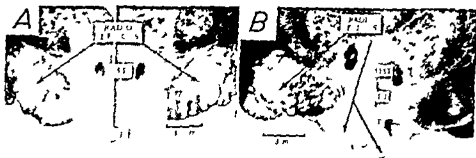


Fig 13 Radiolesions in the brain lateral to the diencephalon

A In Snowflake bilateral destruction in the region of the basal ganglia and internal capsule by radiolesions was associated with aphagia but not adipsia No marked motor disturbance was observed MT mammillothalamic tract MB mammillary body Stain Loyez

B In Ingo radiolesions caused bilateral destruction in the cerebral peduncles and unilateral injury to the internal capsule and lateral hypothalamus Motor and postural disturbance resulted (Fig 11) but no impairment in alimentation occurred F fornix III third ventricle Stain Loyez

D *Histology of goats with hypothalamic radiolesions*

Brains All three goats in this series displayed radiolesions in the median eminence In "Snowflake and "Esmeralda the lesions were found to extend along the midline from the optic chiasm caudally to the mammillary bodies (Fig 12 A) while in Ingo the lesion was situated in the caudal median eminence at the level of the pituitary stalk and extended into the mammillary bodies The two goats which became aphagic but not it should be noted adipsic) had in addition radiolesions in the region lateral to the hypothalamus In "Snowflake , these lesions caused bilateral destruction in the region of the

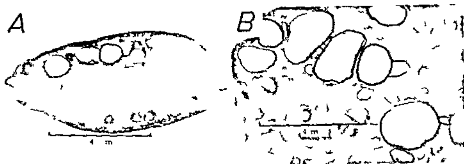


Fig 14 *Effect of median eminence radiolesions on ovaries*

A In Snowflake the ovaries were atrophied and lacked developing follicles thus suggesting a deficiency in secretion of pituitary gonadotrophins had resulted from the hypothalamic damage Stain hematoxylin and eosin

B Ovary from a control goat White which received non-destructive irradiation in the median eminence does not show these changes Stain hematoxylin and eosin

internal capsule and basal ganglia (Fig 13 A) while in Esmeralda smaller lesions were located in the vicinity of the lateral hypothalamus Further both of these goats had lesions in the optic chiasm with possible secondary degeration in the optic tracts The third animal Ingo exhibited bilateral lesions in the cerebral peduncles which apparently accounted for its postural and motor disturbances and an extensive lesion extending from the mammillary bodies unilaterally into the lateral hypothalamus and internal capsule (Fig 13 B)

Pituitaries Histological examination of the pituitary glands of goats with median eminence radiolesions revealed atrophic changes in the posterior lobes absence of marked change in the intermediate lobes and possibly small areas of infarction in the anterior portion of the adenohypophysis (Fig 12 B) This questionable infarction was possibly the result of anoxic ischemia secondary to interruption of hypophysial portal vessels since this portal system comprises the main vascular supply to the anterior lobe in the goat (DANIEL and PRICHARD 1958)

Ovaries The ovaries of these animals showed atrophy and absence of developing follicles most pronounced in Snowflake (Fig 14 A and B)

Discussion

The present finding that radiation induced hypophysectomy blocked milk synthesis in goats during the period in which they remained in good health and in nutritional balance clearly demonstrates the essential role played by the hypophysis in lactation in this species Restoration of milk production up to 60 per cent of pre lesion values was rapidly achieved in goats with pituitary destruction by brief replacement with prolactin STH ACTH triiodothyronine

and insulin The development of aphagia secondary to trigeminal damage, however, prevented a detailed analysis of the proper balance of hormones necessary for full restoration Presumably more complete recovery in lactation would have been attained on this therapeutic regimen if the animals had remained in good health inasmuch as COWIE and TINDAL (1961) have been able to restore milk production more completely in surgically hypophysectomized goats by giving a similar replacement regimen More recently these same workers have been able to restore lactation completely in hypophysectomized goats by administering a therapeutic regimen containing in addition to other hormones 600 I U of prolactin daily, i e, twice the prolactin dosage in the present study (cf FOLLEY 1961)

The present observation that median eminence radiolesions block milk production in goats confirms the effect of surgical injury to the median eminence reported in one goat (TVERSKOI 1960) and of electrolytic destruction of this region in the albino rat (McCANN MACK and GALE 1959) In the present study, the administration of prolactin *per se* to goats with median eminence leasions *could not be shown to improve milk production regardless of whether this* hormone was given alone or in combination with other hormones This finding is in agreement with results from studies of rats with electrolytic median eminence lesions in which lactational impairment was corrected by replacement therapy excluding prolactin (GALE *et al* 1961) These data therefore, suggest that in both species destruction of the median eminence failed to block prolactin secretion appreciably and that lactational imparment resulted from insufficiency of other adenohypophysial hormones Results from the present replacement study suggest that deficiency in pituitary secretion of ACTH TSH and STH caused the decline in milk synthesis It is well documented that median eminence injury interfers with secretion of ACTH (McCANN 1953 and many others) and of TSH (recently in the goat by ANDERSSON *et al* 1963) The effect of such hypothalamic lesions on STH secretion, however is less well understood (REICHLIN 1960) The failure of post milking injections of oxytocin to augment significantly the amount of milk removable showed that oxytoxin deficiency in these goats did not cause lactation to decline by interferring with milk removal

The absence of extensive central necrosis in the anterior lobes of goats with median eminence radiolesions suggests that the vascular supply to the adeno hypophysis was not abruptly interrupted This finding is in sharp contrast to the effect in this species of surgical section of the pituitary stalk, a procedure which causes massive central necrosis in the adenohypophysis by blocking blood flow in the hypophysial portal vessels (DANIEL and PRICHARD 1958) Lactational impairment in the present study therefore, appears to be due to the interruption of regulatory stimuli descending from higher centers rather than due to non specific depression of the adenohypophysis secondary to ischemia It is possible that the slow rate of development of radiolesions impinging on the portal

vessels in the median eminence permitted some compensatory adjustment in the vascular supply perhaps via the short portal vessels from the neurohypophysis

The results of the present study may best be reconciled by the hypothesis that the central nervous system regulates prolactin secretion by means of an inhibitory mechanism, while central control of certain other adenohypophysial hormones e g ACTH (GUILLEMIN *et al* 1959) and LH (McCANN TALEISNIK and FRIEDMAN 1960) is effected by stimulatory influence Since it is now widely believed that the hypothalamic control of anterior lobe secretion is mediated via neurohumors transported down the hypothalamico-hypophysial portal vessels (HARRIS 1955) central inhibitory control of prolactin requires the postulation of a neurohumor with inhibitory action Although no direct experimental evidence has yet been reported on the nature of the postulated inhibitory neurohumor it has been suggested in view of the fact that prolactin and LH are secreted reciprocally (as shown in the albino rat during lactation (McCANN TALESNIK and GRAVES 1961) that the hypothalamic inhibitor for prolactin may be the same neurohumor that has recently been shown to stimulate secretion of LH (McCANN *et al* 1960) An alternative theory that prolactin secretion may be stimulted by the direct action of oxytocin on the adenohypophysial acidophil cells suggested earlier by BENSON and FOLLEY (1957) was based mainly on the evidence that mammary involution could be delayed by injections of oxytocin Since however oxytocin may exert this effect directly at the level of the mammary gland (and not necessarily via the pituitary) (MEITES and HOPKINS 1961) and in view of the considerable evidence supporting inhibitory control of prolactin (see below) this alternative theory no longer seems likely

Various operative procedures which would tend to release the pituitary gland from central inhibition by interrupting the hypothalamico hypophysial connections have been shown either to initiate the secretion of prolactin or to fail to block secretion in progress For example in addition to the lactation study in rats by GALE *et al* (1961) cited above the placement of electrolytic lesions in the median eminence of rats during early gestation (GALE and McCANN 1961) and during the estrous cycle (McCANN and FRIEDMAN 1960) and of unmated rabbits (HAUN and SAWYER 1960) has been found to be compatible with the secretion of prolactin in sufficient amounts to support respectively pregnancy uterine deciduomata and the initiation of milk secretion Similarly the ability of the adenohypophysis to synthesize prolactin persists following transplantation of the gland beneath the renal capsule in the rat as shown by maintenance of uterine deciduomata (EVERETT 1956) and pregnancy (EVERETT 1958) and of lactation (COWIE TINDAL and BENSON 1960) That prolactin secretion can continue in the total absence of central stimulation has been convincingly demonstrated in studies of adenohypophyses incubated *in vitro* in such instances prolactin is synthesized daily in amounts up to ten

times the initial parenchymal content for periods up to six days (NICOLL and MEITES 1962) Further the persistence of prolactin secretion after pituitary stalk section as indicated by maintenance of secreting mammary acini in rabbits (JACOBSOHN 1949), suggests an explanation for a clinical report that milk secretion is initiated in women following stalk sectioning in treatment of cancer (ECKLES EHNI and KIRSCHBAUM 1958) Taken together, these lines of evidence provide strong support for the concept of central inhibitory regulation of prolactin secretion

Diabetes insipidus developed in goats concurrently with the loss of the milk ejection reflex ten to 35 days after irradiation of the median eminence Of particular interest was the finding in these animals that diabetes insipidus commenced without manifestation of the normal interphase, i e the brief period, after the initial increase in water turnover, when water metabolism returns to normal levels and which is followed by the permanent phase of polyuria and polydipsia This normal interphase is believed to result from the release of stores of antidiuretic hormone from the neurohypophysis as that structure undergoes degeneration secondary to interruption of the supraoptico-hypophysial tracts (O CONNER 1952) It seems quite likely that both the delayed onset of diabetes insipidus and the absence of the normal interphase in irradiated animals was the result of the slow development of the radiolesions Thus the atrophy of the neurohypophysis clearly seen at histology must have occurred so gradually as to permit a continuous release of small amounts of stored antidiuretic hormone Upon total depletion the permanent phase of diabetes insipidus would commence This unusual type of diabetes insipidus is in sharp contrast to the classical experimental syndrome of FISCHER INGRAM and RANSON (1935) with its well defined normal interphase and which can be produced in goats by rapid coagulation of the median eminence (GALE 1963) When hormonal replacement was given to goats with diabetes insipidus in the present study the elevated water turnover was augmented further — with the exception of the goats receiving prolactin in the therapy The observed antidiuretic activity of prolactin injections cannot necessarily be attributed to an inherent action since no assay for possible neurohypophysial hormone contaminants was performed ACTH and triiodothyronine appeared to be the hormones administered which were responsible for the further augmentation in water turnover These findings suggest that diabetes insipidus can be sustained in the "hypopituitary goat but that adenohypophysial hormones are required for its full manifestation

Although the ideal situation of restricting radiolesions precisely to the pituitary gland or to the median eminence was not achieved in this study, a similar irradiation technique has been employed successfully by others to destroy the hypophysis in monkeys (SIMPSON *et al* 1959) without causing damage to the CNS It follows therefore that when the relation between distribution of absorbed dose and histological damage are better understood it should be

entirely possible to induce radiolesions in the CNS delimited to the target structures Citing some of the advantages inherent in the irradiation technique over conventional means of producing central lesions may serve to illustrate the usefullness of this method First, the experimental animal experiences practically no stress since no surgery is performed second it is possible to produce larger brain lesions compatible with survival (e g , in the preoptic region /ANDERSSON and LARSSON 1961/) because the radiolesions develop gradually without causing excessive hemorrhage or edema and third the investigator is afforded the opportunity to study the effects of CNS damage developing gradually over weeks and months rather than be witness to the aftermath of sudden central destruction complicated by the effect of hemorrhage and edema on adjacent structures

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Method for Non-destructive Determination of the Sodium Transport Pool in Frog Skin with Radiosodium

By

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Received 1 March 1963

Abstract

ANDERSEN B and K ZERAHN *Method for non destructive determination of the sodium transport pool in frog skin with radiosodium* Acta physiol scand 1963 59 319—329 — A method is described for measuring the amount of sodium confined between an outer membrane which is passively but specifically permeable to sodium and an inner membrane where the active transport mechanism is located The method allows correction for sodium outside the transport pool Several determinations can be performed on the same skin Repetitive determinations gave a standard deviation of ± 7 per cent A single determination can ordinarily be made in less than an hour Good agreement is found under suitable conditions between the potential difference calculated from the pool and the measured potential difference across the skin Furthermore from the disappearance rate of sodium from the pool and the size of the pool the active sodium transport can be calculated under conditions where other methods cannot distinguish between active and passive movements of sodium

Since the discovery of active ion transport across biological membranes many theories have been advanced as to the mechanisms of the transport processes (for references see USSING 1960)

In the case of active sodium transport experiments seem to support the idea of a forced exchange of sodium for potassium in the membrane as being part of the transport mechanism (STEINBACH 1952 HODGKIN and KEYNES 1955 KOEFOED JOHNSEN and USSING 1958) In the theory advanced by KOEFOED JOHNSEN and USSING (1958) for sodium transport through frog skin the sodium ions are supposed to pass through the cells and hence have to penetrate at least two cell membranes viz the outward facing and the inward facing membranes of the epithelial cells of the frog skin Whereas the outward fa-

cell membrane lets sodium ions through in a selective but purely passive way, the active transport takes place at the inward facing membrane by way of the forced exchange of sodium for potassium with a concomitant expenditure of metabolic energy So far almo t all investigations on transcellular transport of ions have been concerned with the influence of external factors on the transport mechanism as a whole Very little has been published about changes in the internal ionic environment of transporting cells in response to external conditions It has been the aim of the present study to develop a method for the determination of the cellular sodium content as part of a more extensive study of the Na transport mechanism proper The method to be presented here is direct and does not necessarily require steady state conditions Furthermore several determinations can be made on the same skin a single determination being performed in 45 minutes

Theory

The method rests on the assumption that sodium in order to be actively transported through the frog skin has to be in some specific compartment i e the epithelial cells This compartment is confined by two boundaries of which the inner most is the site of the active sodium pump (KOEFOED JOHNSEN and USSING 1958) Furthermore it is assumed that the transport pool consists of sodium from the outside solution This implies that the pathways of efflux and of influx through the skin are separated This assumption will be discussed in a future paper from this laboratory

The principle of the transport pool determination is as follows

The outside solution is labeled with Na 22 or Na 24 and isotopic equilibrium is awaited Now the outside solution is completely removed and the residual radiosodium in the skin is measured with time This is done by placing a G—M counter above the skin and measuring the radioactivity (Fig 1 B) Since the outside is dry the radiosodium can only escape to the connective tissue by action of the sodium pump Although a small fraction of the labeled sodium will be caught by cells in the connective tissue, most of the sodium transferred to this tissue will diffuse into the inside solution with a half time of less than a minute (HOSHIKO and USSING 1960) Furthermore radiosodium in the connective tissue will be measured with a lower efficiency due to selfabsorption Once in the inside solution the radiosodium is flushed into a big reservoir and hence does not contribute to the activity measured by the G—M counter

At zero time the radiosodium in the skin is mainly contained in the active transport pool located in the epithelial layer Determination of the size of the pool from radioactivity measurements of course requires that exchange diffusion or back diffusion through the inner membrane of the pool is insignificant

If, however the transport mechanism is located in the outer layer of the epithelium the measured pool will consist at least in part of labeled sodium already transported At the time being this possibility can not be excluded

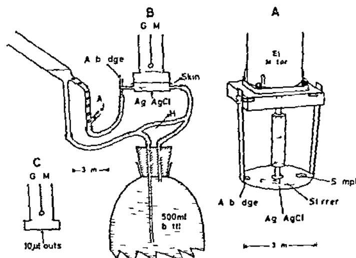


Fig 1 A Set up fo loading the skin ith radiosodium B Set up for following the disappear ance of rad osodium C Set up for measuring activity of standard G-M G M counter and scaler A bridge Agar agar bridge for measuring potential over skin Ag AgCl Chlorinated silver plates H Shunt for the determination accord ng to Hoshiko and Ussing 10 µl outs 10 µl outside Na 22 solution distributed evenly on lens paper with 25 µl dextrose solutio i Sample Hole for sampling Stirrer Stirrer made of perspex.

Technique

The ventral skin of *Rana temporaria* or *Rana arvalis* was placed in the apparatus (see Fig 1) the epithelial side facing upwards The inside solution was Na R Na_2SO_4 R or Li R aerated and circulated in the usual way The composition of the solutions was as follows

Na R NaCl 115 mM, $KHCO_3$ 2 5 mM $CaCl_2$ 1 0 mM

Na_2SO_4 R Na_2SO_4 57 5 mM $KHCO_3$ 2 5 mM $CaSO_4$ 1 0 mM

Li R Li Cl 115 mM $KHCO_3$ 2 5 mM $CaCl_2$ 1 0 mM

The outside solution contained sodium in various concentrations labeled with radiosodium For short-circuiting experiments the normality of the outside and inside solu tions were equal The potential bridges were prepared from a solution which contained 3 per cent agar agar in either the same solution from which the potential was to be measured or saturated KCl The current electrodes were silver silverchloride when chloride was the major anion in the solution When sulphate was used as anion, platinum electrodes were used in short periods electrolysis will be small and the pH will remain fairly constant The volume of the outside solution was 3—5 ml the volume of the inside solution was about 600 ml

Procedure

The general procedure was as follows The skin was mounted and adjusted to the experimental conditions wanted Next the outside solution was changed to the labeled solution or the radiosodium was added in a small volume and isotopic equilibrium across the outer boundary was awaited Usually 10 min was sufficient, but with low fluxes of sodium up to 50 min was necessary At the time $t = 0$ the upper current

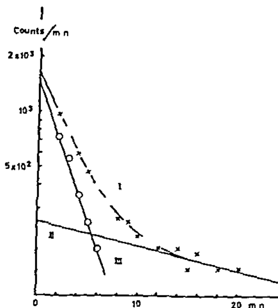


Fig 2 Na 22 disappearance from skin of *Rana temporaria* Curve III is derived by subtracting values of the curve II from the found values curve I

electrode was removed together with the stirring device the outside solution was removed as quickly as possible the skin was washed twice with 2 ml of distilled water and blotted with soft paper The G—M end window counter was placed in position and measurements were taken every minute The readings were plotted in a semilog graph Fig 2 The first part of the curve is a straight line and can be extrapolated to time zero After some time the curve changes its slope and eventually becomes another straight line Curve I can be resolved into two straight lines II and III As will be discussed later curve II represents radioactivity from other sources than the transport pool The other straight line III gives the amount of radiosodium in the transport pool at any time The extrapolated value on the ordinate is counts per minute and can be converted into μ eq of Na This is done by counting the radioactivity of 10 μl of outside solution under conditions which will give practically the same geometry back scattering and self absorption as the epithelial layer (see Fig 1)

Discussion

Before discussing the significance of the determined pool some comments have to be made about the experimental procedure

1) Washing

Since the outside solution cannot be removed completely by blotting it is necessary to know whether a quick washing of the outside will remove Na from the transport pool In earlier experiments by one of us (K Z) the amount of solution still adhering to the skin after blotting was determined with Ca-45 18 determinations gave a mean value of 6 7 μl (range 4 7 to 11 2) In the present work the amount of adhering solution has been estimated from the difference in apparent pool of the same skin when it is just blotted and when it is both

Table I Comparison between calculated active Na flux and short circuit current in skins of Rana temporaria bathed in Ringer's Area of skin is 7 cm²

Calculated flux (μEq/hr)	Current (μEq/hr)
17 ?	11 3
13 7	10 1
18 5	17 1
5 0	6.5
11 5	10 8
14 9	11 3
10 0	13 4
6 9	8 1
8 0	10 8
8 6	9 7
Mean value 11 4	10 9

washed and blotted 13 determinations gave a difference of 5 1 μl (range 2 5 to 8 1) Since the mean values obtained by the two methods are practically identical there can be no significant loss of sodium from the skin to the washing fluid

With a low outside Na concentration it is of no importance whether the skin is only blotted or washed because the pool is representing many μl of outside solution

2) Time for isotopic equilibrium

A preliminary experiment yields an approximate half time Usually a loading period of 5 times this half time is practical In many cases 90 per cent equilibrium will suffice because an error of 10 per cent is not important

3) Zero time

In short circuit experiments we have taken the moment when the short circuit current is cut off by lifting the upper electrode to be zero time In experiments with the spontaneous skin potential we found it natural to choose as zero time the moment when the outside solution was removed by washing However the difference in time will be only about 15 sec causing a difference in the estimated pool of 5 to 10 per cent

4) Transport pool and apparent pool

Curve I in Fig 2 gives a certain value for the Na pool This apparent pool includes however sodium which is not part of the transport pool for which due corrections must be made To determine where the radiosodium is localized the slicing technique of Hvid Hansen and Zerahn (1963) has been used It was found that hardly any residual activity was located in the epithelial layer after 20 min thus curve II can be correlated with the activity found in

Table II Repetitive determinations of the transport pool under constant conditions The skins were short circuited and bathed in Ringer s Area of skin is 7 cm

Current (μAmp)	Pool (μEq Na)
258	0 87
290	0 83
40?	0 76
452	0 93
390	0 85
422	0 77
480	0 87
465	0 79
400	0 89
385	0 77
365	0 74
475	0 92
488	1 04
485	0 98

the cellular components of the connective tissue at the edges of the skin and the upper ring of the plastic apparatus This activity is obviously not part of the active transport pool

Attempts to localize sodium quantitatively just after loading were given up due to the short half time for the transport pool but preliminary slicing experiments showed that even 2 min after t = o about 50 per cent of the total activity in the skin is still located in the epithelial layer

The intervals between reading the accumulated counts were set to 1 min This is a long time compared to the half time for the transport pool, but the actual error commited is insignificant The large volume of the inside solution makes the disturbances from the activity of the inside solution negligible From the size of the transport pool and the half time the flux of sodium can be calculated by the following formula

$$\text{Initial flux of Na ions per hour} = \frac{\text{pool at } t = o}{t_{1/2}} \times 60 \ln 2$$

where $t_{1/2}$ is the half time in minutes read from curve III (see Fig 2) In short circuit experiments this flux can be compared with the short circuit current In Table I it can be seen that there is fair agreement

5) Reproducibility

In Table II are given repetitive determinations of the transport pool under constant conditions (on the same skin) The reproducibility was calculated to be ± 7 per cent

Table III Comparison between half times ($t_{1/2}$ Na and $t_{1/2}$ Li) for sodium transport pool with sodium or lithium as the inside cation Outside Na = 115 mM Species Rana temporaria

$t_{1/2}$ Na	$t_{1/2}$ Li
2 9	2.2
2 6	2.7
3 3	3 0
3 6	3.2
2 7	3 1
3.5	3 6
Mean value 3 1	3 0

6) Exchange diffusion back diffusion

For definition of the concept of exchange diffusion across the inner membrane where the sodium pump is located we have adopted the definition given by USSING (1948) By back diffusion we mean diffusion from the inside solution into the transport pool

If back diffusion in this sense or exchange diffusion were of significant magnitude the specific activity of the sodium in the transport pool would be lower than that of the sodium in the outside solution Thus the pool measured by the present method would be too low

A number of arguments may however be advanced against the occurrence of such processes

The efflux of sodium (i e passage from the inside to the outside solution) observed in skins by addition of radiosodium to the inside solution might take place either through the transporting cells starting with back diffusion or exchange diffusion through the inner membrane or through routes by passing the transporting cells In the former case a change in the sodium permeability of the outer cell membrane should lead to a change in the sodium efflux However several experiments have failed to demonstrate such changes In *Rana esculenta* it is reasonable to assume that atropine (KIRSCHNER 1955) and procain (SKOU and ZERAHN 1959) increase the sodium permeability of the outer membrane without affecting the transport mechanism at the inner membrane In spite of an increase in sodium influx the efflux remains constant within the experimental accuracy

In *Rana temporaria* calcium ions on the outside decrease sodium influx most likely by decreasing the sodium permeability of the outer membrane (CURRAN and GILL 1962) Efflux remains however essentially unchanged We have found that Li ions in the outside solution decrease the Na permeability of the outer membrane without effect on the transport mechanism at the inner membrane From a paper by ZERAHN (1955 Table I) it can be seen that if due corrections are made for the decrease in sodium concentration in the inside

Table II PD measured and calculated according to Koefoed Johnsen and Ussing (1958) for isolated frog skin in sulphate Ringer s [K] taken as 100 mM Subscripts c and o refer to concentrations in epithelial cell inside and outside solution respectively

Na	Na	K	Calc PD	Found PD	Species
0 23	0 32	115	−12	−30	Rana arvalis
0 23	0 39	115	−17	−2?	
2 3	1 35	115	10	0	
2 3	2 0	115	0	− 3	
0 ?3	0 42	115	−19	−20	
115	?2 8	115	38	17	
115	9 3	115	61	40	
115	14 1	115	50	15	
115	13 6	115	51	51	
115	21 3	115	40	49	
115	24 3	115	36	23	
115	7 8	2 5	164	146	
115	11 7	2 5	154	144	
115	9 3	2 5	159	165	
115	7 5	2 5	164	101	
0 23	0 23	2 5	95	17	
0 23	0 27	2 5	90	9	
1 15	1 14	38	25	21	Rana temporaria
115	8 4	115	63	40	
115	10 8	115	57	21	
115	13 5	115	51	24	
115	8 1	2 5	168	89	
115	7 2	2 5	166	47	
115	5 4	2 5	173	132	
115	8 4	2 5	16?	128	
1 15	1 08	2 5	96	57	

solution sodium efflux remains constant wether or not Li is present on the outside It can therefore be concluded that efflux through transporting cells is insignificant and thus back flow of sodium through the cell membranes does not constitute a significant error in the determination of the sodium transport pool

Correspondence between the sodium flux calculated as mentioned above, and the short circuit current cannot be used as an argument against the existence of exchange diffusion since in the presence of this process both the transport pool and the half time would be underestimated to the same extent However a strong argument against the existence of a significant exchange diffusion rests in the fact that replacement of sodium in the inner solution with other cations — lithium potassium or choline — were found to cause no significant changes in the half time observed in successive measurements on the same skin The values obtained in some lithium substitution experiments

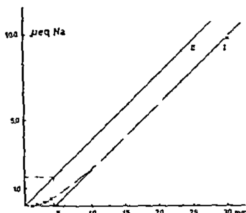


Fig 3 Curve I is the sodium derived from the outside (labeled Na) which actually appears on the inside Curve II is obtained by drawing a line parallel to the straight part of I through the zero point. The final vertical difference A between I and II is the amount of labeled Na used to bring the skin from an unlabeled steady state to the labeled one

are presented in Table III These findings contrast with those made by KEYNES and SWAN (1959) in muscles In their experiments an appreciable decrease in the disappearance rate of labelled sodium in frog muscles was observed upon substitution of lithium for sodium in the bathing solution This is equivalent to an increase in half time thus indicating the existence of sodium exchange diffusion across the muscle cell membrane

Frog skin will not always tolerate Li R on the inside for prolonged periods but so far there has been no effect of lithium on short circuit current in a period long enough to determine half time

7) Calculation of skin potentials according to KOEFOED-JOHNSEN and USSING (1958) from transport pool

If the concentration of sodium and potassium in the transporting cells is known the potential difference can be computed provided that there is no short circuiting by other ions From slicing experiments (HVID HANSEN and ZERAHN 1963) the potassium concentration can be estimated to be between 100 and 140 mM when corrected for dry matter and extracellular space For the present purpose the concentration has been set at 100 mM The sodium concentration is derived from the apparent transport pool by setting the volume of the epithelial layer to 5 $\mu l/cm^2$ Both the apparent pool and the volume are too large The errors introduced will at least partly cancel each other The accuracy will however be sufficient since a factor of 2 will only show as 20 mV Now the potential can be calculated according to the following formula when only non penetrating anions are present in the outside and the inside solutions

$$E\ (mV) = 58 \log \left(\frac{K}{K_i} \times \frac{Na}{Na}\right)$$

where the subscripts c i and o refer to the concentration of the ion in question

Table V Na pool in μEq/7 cm² frog skin determined by different methods (see section 8)

Kinetic method	Graphic method	Present method
0.84	1.25	0.61
0.60	0.68	0.40
0.52	0.70	0.31
1.38	1.85	1.01
1.51	1.55	0.97

in cells, inside and outside solution, respectively In Table IV the PD calculated in this way is compared with that actually measured There is satisfactory agreement between calculated and found values for the PD when inside [K] is high and outside [Na] is low This is reasonable because the potential differences across the membranes are low and consequently the short circuit from other ions limited In some cases especially with *Rana arvalis* the natural potential is very high (above 140 mV) and good agreement is obtained with all concentrations of potassium and sodium

8) Comparison with other methods

A kinetic determination of the sodium pool of the transporting system of the frog skin has been treated by Hoshiko and Ussing (1960) under the assumption that influx and efflux are using the same route Experiments from this laboratory (to be published) indicate however, that extracellular pathways exist

Another way of obtaining an evaluation of the pool is to plot the accumulated sodium flux versus time An example is given in Fig 3 At zero time the skin is in a steady state and the radiosodium is added to the outside solution The labeled sodium appearing on the inside is measured and plotted with time all the sodium appearing and accumulated on the inside for each point When curve I (Fig 3) is a straight line the steady state for the labeled outside sodium is reached and the difference in height between curve I and curve II will give the amount of labeled sodium retained in the skin and used for bringing the skin to the labeled steady state

These two methods both give the total pool and provide no means for correction for radiosodium which is not in the transport pool Therefore these determinations will give values which are somewhat larger than does the present method In these methods the problems with exchange diffusion and back diffusion are the same as in the present method In Table V sodium pool determined by the two above mentioned methods and the present method is compared

As mentioned the method rests on the assumption that the sodium transport takes place at the inner cell layer of the epithelium If however the transport

takes place at the outer cell layer the significance of the measured pool and half time will be quite different In this case the half time is a measure of the diffusion resistance for sodium from the site of the active transport to the inside solution and does not reflect the efficiency of the sodium pump Although the pool will be mainly a *transported* pool identity between short-circuit current and calculated sodium flux will still hold Further experiments are needed before the exact location of the sodium pump can be decided upon

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Localization of Radioactivity in the Superior Cervical Ganglion of Cats Following Injection of C^{14}-labelled Nicotine

By

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Abstract

APPELGREN L E E HANSSON and C G SCHMITERLOW *Localization of radioactivity in the superior cervical ganglion of cats following injection of C^{14} labelled nicotine* Acta physiol scand 1963 59 330—336 — The distribution of (—) nicotine methyl C^{14} in the superior cervical ganglion of the cat was studied by using autoradiographic and microautoradiographic techniques Five minutes after the intracarotid injection of labelled nicotine the ganglion was removed frozen instantaneously and then prepared for autoradiography From the microautoradiograms presented it becomes evident that almost all of the radioactivity is localized to the ganglion cells whereas the satellite cells and the connective tissue contain very little radioactivity It is noteworthy that some of the ganglion cells contain much more radioactivity than others The possible explanation for this discrimination is discussed

In earlier papers (SCHMITERLOW and HANSSON 1962 HANSSON and SCHMITERLOW 1962 and APPELGREN HANSSON and SCHMITERLOW 1962) we have described the distribution and metabolism of C^{14} labelled nicotine It was found that the brain rapidly accumulated nicotine and that initially the nicotine was concentrated to the parts of the brain rich in nerve cells We also found a high concentration of nicotine and/or its metabolites in the suprarenal medulla

Since one of the most conspicuous pharmacological actions of nicotine is on the autonomic ganglia we thought it of interest to study the localization of C^{14} labelled nicotine in such a ganglion Autoradiographic and microautoradiographic methods were employed

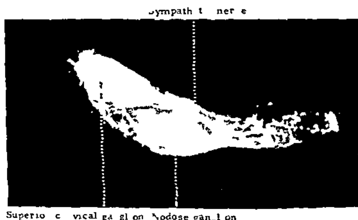


Fig 1 Autoradiogram of a longitudinal section of the superior cervical ganglion and the nodose ganglion with their adjacent nerves (cat) 5 minutes after intracarotid injection of C^{14} nicotine White areas correspond to radioactivity ×10

Methods

Synthesis of (—) nicotine methyl C^{14}

Nicotine methyl C^{14} was synthesized by methylating (—) nornicotine using formaldehyde C^{14} in the presence of formic acid (McKENNIS *et al* 1961) as described earlier by HANSSON and SCHMITERLOW 1962 The C^{14} nicotine used throughout the present investigation belonged to the same batch as the C^{14} nicotine used in our previous work (APPELGREN *et al* 1962) The specific activity of the (—) nicotine methyl C^{14} was 78 μC/mg

Animal experiments

Cats (approximate weight 3 kg) were used under Mebumal® anesthesia The trachea was cannulated All branches of the common carotid artery were tied excepting those supplying the superior cervical ganglion Clotting was prevented by intravenous injection of 200 units of heparine (Vitrum) per kg body weight A needle 27 gauge connected to a polythene tube was inserted into the common carotid artery for injection of C^{14} nicotine In order to ascertain that nicotine reached the ganglion and exerted its proper effects the behaviour of the nictitating membrane following preganglionic electric stimulation was studied

The amount of C^{14} nicotine injected was 0 5 mg corresponding to approximately 40 μC The C^{14}-nicotine was dissolved in physiological saline and the volume injected was 0 2 ml

The superior cervical ganglion was removed 5 min after the injection of C^{14} nicotine After having been removed the ganglion was divided into three pieces These were frozen instantaneously by immersion into iso-pentane cooled with liquid nitrogen They were then transferred to a tissue holder for freeze-drying The freeze-drying was performed in a modified Glick Malmstrom apparatus (MOBERGER *et al* 1954) The freeze drying was continued for 24 hours after which the ganglion pieces were embedded in paraffine In some control experiments we made certain that no radioactivity was extracted from the tissue pieces into the paraffine

The following experiment was carried out in two kittens weighing approximately 0 75 kg After tying off the main branches of the common carotid artery and injection of



Fig. 2 Micro-autoradiogram from the superior cervical ganglion (cat) 5 minutes after intracarotid injection of C nicotine Film and section tog ther Black spots (sil er grains) indicate the deposition of radioactivity
Staining Hemalum-eosin 80

C^{14} nicotine as mentioned above the superior cervical ganglion the nodose ganglion and the adjacent nervetrunks were cut out (5 min after the injection of C^{14} nicotine) This preparation was immersed into iso-pentane cooled with liquid nitrogen without cutting the ganglia into pieces The ganglia with their nerves wer then mounted on a microtome stage

Histological and autoradiographical methods

Seven micron thick sections were made from the freeze-dried ganglion pieces In order to obtain whole sections without floating them on water — which would result in the dissolution of the water soluble C^{14} nicotine and/or its metabolites — Scotch cellulos tape was carefully pressed on to the flat upper surface of the block before cutting the section The sections on tape were then mounted with a small amount of egg albumen glycerol on Kodak AR 10 stripping emulsion already stripped on glass slides subbed with chromealum gelatine After exposure for about 30 days the tape was removed by immersion of the whole specimen in xylene for 48 hours The xylene was removed with absolute alcohol In order to make the penetration to the film of developer and fixer possible the plates were passed through 95 per cent alcohol for 2 min and 70 per cent alcohol for another 2 min The film was developed in Kodak developer D 19 and carried out for 5 min at 20 C The preparations were then fixed in an ordinary acid fixer used for X ray films After thorough washing the sections on the film were stained with hemalum-eosin and the whole preparation (section on film mounted in Euparal (trade mark, Flatters & Garnett Ltd Manchester England) Using this technique the section and the film are together in close contact with each other throughout the whole procedure Microscopic examination will thus show the stained section on top of the film where silver grains mark the presence and position of the radioactive tracer The photo-



Fig 3 Enlargement from Fig 2 Note that the radioactivity is concentrated in the ganglion cells ×360

graphic pictures presented here are slightly blurred because of the fact that the microscope has to be focussed somewhere between film and section

The whole ganglia with their adjacent nerves removed from the kittens were sectioned and autoradiographed according to the method described by ULLBERG (1954)

Results

Gross distribution

Fig 1 shows an autoradiogram of the superior cervical and nodose ganglia with their adjacent nerves The intracarotid injection of C^{14} nicotine was made 5 min before removal and freezing the ganglia As can be seen there is an accumulation of radioactivity in the sympathetic ganglion whereas the amount of radioactivity is much less in the nodose ganglion It is also obvious that the sympathetic ganglion contains more radioactivity than the symphathetic preganglionic nerve trunk The fact that the capsule of the nodose ganglion seems to contain a high amount of radioactivity is probably due to the leakage of blood containing C^{14} nicotine when the ganglia are removed

Microautoradiographic investigations

Fig 2 shows the distribution of radioactivity at a cellular level in the superior cervical ganglion It is noteworthy that in this survey microautoradiogram it seems as if some of the ganglion cells contain much more radioactivity than others Fig 3 is an enlargement from the section + film shown in Fig 2 (area inside the outlined rectangle) When looking at these two microautoradiograms it becomes evident that almost all of the radioactivity is localized to the ganglion cells whereas the satellite cells and the connective tissue contain very

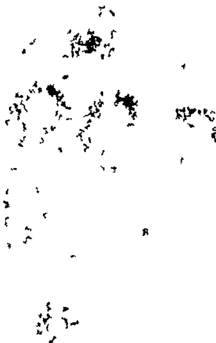


Fig. 4. Microautoradiogram from the superior cervical ganglion (cat) 5 minutes after intracarotid injection of C¹⁴-nicotine. Film and section together. Black spots (silver grains) indicate the deposition of radioactivity. Staining: hemalum-eosin. × 360.

radioactivity. It can also be observed that in many cases the radioactivity (corresponding to the black silver grains) seems to be concentrated to one pole of the ganglion cells. The large ganglion cells seen in Fig. 3 and 4 demonstrate this clearly. These two figures show that the radioactivity is mainly concentrated in the peripheral parts of the ganglion cells. It is impossible to tell whether the radioactivity is concentrated within the cells or if it is located on the surface of these cells. The microautoradiograms shown in Fig. 2—4 all show the distribution picture 5 min after the intracarotid injection of C^{14}-nicotine.

Discussion

The now classic work of Langley and his co-workers at the turn of the century revealed the action of nicotine on autonomic ganglia. The mechanism behind the initial stimulating action followed by an inhibition of synaptic transmission has been subject to many different interpretations (cf. PATON and PERRY 1953, LUNDBERG and THESLEFF 1953, PELIKAN and GARCIA 1959, PELIKAN 1960). In the present investigation we only show by visual demonstration that nicotine actually concentrates to the ganglion cells of the superior cervical

ganglion There must obviously be some sort of selective mechanism by which nicotine is directed to the ganglion cells

The autoradiograms shown in this paper all derive from ganglia removed 5 min after the intracarotid injection of C^{14} nicotine It is a well known fact that nicotine is fairly rapidly metabolized in the body Due to the short time interval between injection and removal and freezing of the ganglia it is likely however that most of the radioactivity shown in our autoradiograms derives from unchanged nicotine If longer intervals had been used more and more of the radio activity would presumably have been present in metabolites from nicotine

Some of the findings should be briefly discussed here

The difference in uptake of radioactivity between the superior cervical and the nodose ganglion might be explained by their different structure and physiological mode of action As the nodose ganglion belongs to the visceral afferent part of the vagus it serves the same purpose as a spinal ganglion It was stated already by LANGLEY (1901) that nicotine has no stimulating or paralyzing action on the spinal ganglia If there is no cholinergic synaptic transmission in the nodose ganglion it seems unlikely that nicotine should accumulate in this ganglion In the superior cervical ganglion on the other hand the accumulation of nicotine is quite in accordance with the view that nicotine interferes with the cholinergic synaptic transmission

Some of the ganglion cells in the superior cervical ganglion accumulate more nicotine than others The reason for such a discrimination is hard to explain It may be that for some reason or other there is an irregular nicotine distribution within the ganglion Another explanation could be that nicotine apart from its interference with synaptic transmission has a specific affinity for those ganglion cells which contain a high amount of acetylcholinesterase Such cells are known to be present in various amounts in different sympathetic ganglia (for references see KOELLE 1962 HOLMSTEDT LUNDGREN and SJÖQVIST 1963 SJÖQVIST 1962 1963 a b) From SJÖQVIST s (1962) investigation it is apparent that only 1 per cent of the ganglion cells in the superior cervical ganglion of the cat belongs to the group of cells which shows an especially high content of acetylcholinesterase (type I cells) Judging from our autoradiograms however the number of ganglion cells with a high amount of nicotine constitutes much more than 1 per cent of the total number of ganglion cells Still another explanation for the difference in nicotine uptake in different ganglion cells refers to the findings of VOLLE (1962 a b) who studying the effects of a number of ganglionic blocking and stimulating drugs on the superior cervical ganglion found evidence for the concept that the sympathetic ganglion cells are functionally heterogenous This finding is in accordance with the hypothesis of functional differences among synapses put forward by SHAW *et al* (1951) and MAINLAND and SHAW (1952)

Experiments showing the distribution of nicotine in ganglia after pretreatment with hexamethonium and after pre ganglionic denervation are in progress

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Acta physiol. scand 1963 59 337—348

From the Neurophysiological Laboratory at the Anatomical Institute University of Oslo Norway

Excitatory and Inhibitory Bladder Responses to Stimulation of the Cerebral Cortex in the Cat

By

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Abstract

GJONE R and J SETEKLEIV *Excitatory and inhibitory bladder responses to stimulation of the cerebral cortex in the cat* Acta physiol scand 1963 59 337—348 — A continuous intravesical pressure recording was established in 22 anesthetized cats by operative canalization of the bladder cavity leaving the urinary outlet intact The intravesical pressure variations caused by controlled volume changes were studied and the alterations in bladder activity in response to stimulation of the cerebral cortex were recorded The following *excitatory* bladder reactions on cortical stimulation are described augmentation of the rhythmic contractions dropwise expulsion of the bladder contents with incomplete emptying of the vesical cavity and finally a normal micturition act with forceful detrusor contractions With regard to *inhibitory* effects the following responses were obtained reduction or complete inhibition of the rhythmic bladder activity arrest of the urination act or suppressed micturition In agreement with previous investigations *excitatory* bladder responses were obtained from the first sensory motor area and from the anterior cingulate region In addition excitatory effects were elicited by stimulation of the anterior ectosylvian and anterior sylvian gyri (the somatic sensory motor area II) Previous observations of *inhibitory* influence on bladder activity from the first sensory motor area were confirmed In addition inhibitory effects were produced from the somatic sensory motor area II the subcallosal part of the anterior cingulate region and the orbital gyrus All the various types of bladder reactions described above were obtained from any of these regions

The influence of the cerebral cortex on the activity of the urinary bladder has been the subject of only a few systematic experimental studies In particular very little is known about inhibition (arrest of micturition and relaxation of the bladder) in response to cortical stimulation The main purpose of this study was to localize the cortical areas influencing bladder motility both with regard to excitation and, in particular inhibition

The first evidence of cortical *excitatory* influence on the bladder function was presented by BOCHEFONTAINE (1876) who observed bladder contractions in the curarized dog on faradization of the cortex surrounding the cruciate sulcus This observation was confirmed in experiments on cats and dogs by BECHTEREW and MISLAWSKY (1888) These workers recorded intravesical pressure elevation in response to stimulation of the medial part of the posterior sigmoid gyrus in the curarized animal Similar effects from the sensory motor cortex were later obtained in the dog by FRANKL HOCHWART and FROLICH (1904) and in the cat by HUNSICKER and SPIEGEL (1933) and LANGWORTHY and KOLB (1935) CROUCH and THOMPSON (1939) observed micturition resulting from stimulation of the same cortical region in cats dogs and monkeys

During the past two decades systematic studies have also revealed excitatory bladder responses from the posterior orbital region in dogs (OKINAKA *et al* 1953) the anterior cingulate area of the cat (STROM and UVNAS 1950 INGERSOLL JONES and HEGRE 1961) the posterior cingulate region in dogs (KREMER 1947) and cats (HENNEMAN 1948) the prepyriform cortex of the dog and cat (KOIKEGAMI *et al* 1957) and the posterior pyriform cortex in cats (HENNEMAN 1948 SMITH 1949) From the latter area KAADA (1951) elicited urination (as well as defecation) in cats dogs and monkeys

Inhibitory bladder responses recorded by a fall of the intravesical pressure, have been elicited from the sensory motor cortex (HUNSICKER and SPIEGEL 1933 LANGWORTHY and KOLB 1935) In addition relaxation of the bladder has been observed by stimulation of the posterior cingulate and pyriform cortex (HENNEMAN 1948)

In the experimental studies referred to above the excitatory and inhibitory bladder responses were recorded as alterations in the intravesical pressure either by the use of a urethral catheter or by operative canalization of the bladder cavity through a cystostomy When the latter procedure was employed the neck of the bladder was ligated to provide a closed cavity, from which no leakage could then occur during the intravesical pressure recording In both cases the micturition reflex was abolished by the suspension of the sphincter mechanism

In order to gain further knowledge about cortical regulation of bladder activity it was considered essential to modify the recording technique so as to include the dynamics of the micturition act thus facilitating a study of the way this act is influenced by cortical stimulation The experimental conditions required may be achieved by utilizing the technique of 'direct cystometry

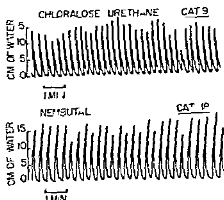


Fig 1 Rhythmic spontaneous activity of cat bladder Above chloralose urethane anesthesia. Below nembutal anesthesia

(MURPHY and SCHOENBERG 1960) By this method the urinary outlet is left fully intact the intravesical pressure readings being obtained through a bladder puncture In the present experiments it was found more convenient to perform a cystostomy maintaining the advantage of an undisturbed sphincter mechanism

Material and Methods

A total of 22 cats of both sexes (weight 1 4—4 2 kg) were used The entire experimental procedure was carried out under general anesthesia usually by intraperitoneal administration of 1 per cent chloralose[1] (40 mg/kg) and 25 per cent urethane (500 mg/kg) Two animals were given paralyzing doses of succinylcholine Curacit© Nyco[1] (1 mg/kg) and ventilated artificially with pure oxygen through a tracheostomy In these cases anesthesia was induced by injections of chloralose alone using the regular dose Three animals were given Nembutal© Abbott (30 mg/kg)

Cystostomy was performed through a midline abdominal incision and a Nelaton catheter No 12 was inserted into the fundus region and sutured to the bladder wall By means of a Statham pressure transducer connected to a Grass polygraph (Model 5 C) with DC-amplifiers a continuous intravesical pressure recording was established Blood pressure and respiratory movements (represented by the excursions of the thoracic wall) were recorded simultaneously on the same polygraph in 9 and 6 animals respectively In the first three experiments the bladder response was obtained by means of the closed system method using an intravesical balloon whereas all subsequent experimental data were based upon the modification of the direct cystometry mentioned above By this technique an isometric recording of the intravesical pressure is obtained as long as no urination takes place In addition the pressure changes during the voiding act were recorded After replacing the urine by saline solution at body temperature, the bladder volume could be checked through the 3 way tube connecting the catheter to the transducer

The animal s head was fixed in a Horsley Clarke frame and a craniotomy performed the cerebral cortex being exposed to the extent required The cerebral cortex was stimulated by electric square waves of 1 msec duration and applied at different fre

[1] Chloralose was kindly suppl ed by E Merck Darmstadt and Curacit by Nyegaard & Co A/S (Nyco) Oslo



Fig 2 Cystometrogram obtained by step-wise filling of the bladder during chloralose urethane anesthesia

quencies (1—100 cps) through bipolar silver chlorided ball tipped electrodes with an interelectrode distance of about 2 mm The stimulus strength necessary to elicit bladder responses was 2—4 volts (0 16—0 32 mA) In mapping the cortical surface intensities of 5—7 volts (0 40—0 56 mA) were ordinarily used Stimulation of the cerebral cortex was not started until 3 hours after the induction of anesthesia

Results

(A) *Background activity*

Rhythmic variations of the intravesical pres ure were constantly recorded in the anesthetized animal These pressure changes representing detrusor contractions in response to distention of the bladder were seen not only during the standard chloralose urethane anesthesia but also when nembutal was used (Fig 1) The frequency and amplitude of the detrusor contractions were in creased by increasing distention of the bladder until finally micturition oc curred The micturition threshold *i e*, the degree of bladder filling necessary to precipitate urination showed individual variations from 10 to 75 ml and was regularly found to be higher in the anesthetized male cats than in the females The bladder capacity seemed to bear no definite relationship to the body weight but was clearly dependent on the type of anesthesia Thus the micturition threshold was significantly higher under nembutal as compared with chloralose urethane anesthesia To demonstrate this phenomenon further 20 mg nembutal kg body weight was added in one experiment to the standard do e of chloralose urethane 3 hours after the administration of the latter The bladder capacity was raised thereby from 10 to 65 ml Under ordinary con ditions this critical intravesical volume did not however vary significantly in one and the same animal throughout the experiment not even during successive cystometries performed by step-wise bladder filling at intervals of 3 to 5 min As shown in Fig 2 each volume increment caused a sharp pressure rise followed by a slow return to a stable tension level The latter represented a very slight elevation of the intravesical pressure as compared with the preceding level

Fig 3 Excitatory stimulation effects on bladder activity and concomitant changes in blood pressure and respiratory movements Stimuli of 1 msec duration were applied at 5 volts to the cortical areas indicated by arrows A Intravesical pressure rise in response to stimulation at a frequency of 50 cps B Dropwise urination elicited at 5 cps C Micturition in response to stimulation at 50 cps

(B) Bladder responses resulting from electrical stimulation of the cerebral cortex

In the summary diagrams of the responsive cortical areas (Fig 8) the stimulation effects are referred to simply as excitatory (↑) or inhibitory (↓) It should be emphasized however that the two symbols represent different types of bladder reaction Thus an excitatory response includes augmentation of the rhythmic detrusor contractions (Fig 3a) as well as precipitation of a voiding act The latter was observed either as an abortive type of micturition with drop wise incomplete expulsion of the bladder contents (Fig 3b) or as sus

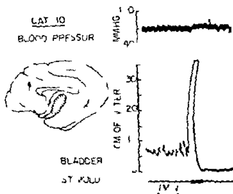


Fig 4 Curarized animal Micturition accompanied by a marked rise in intravesical pressure resulting in complete emptying of the bladder Point indicated by arrow stimulated with 4 volts at 50 cps

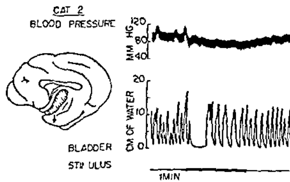


Fig 5 Inhibition of the rhythmic bladder activity and concomitant fall of blood pressure elicited from the pyriform cortex using 5 volts and 50 cps

tained detrusor contractions with complete emptying of the vesical cavity (Fig 3c and Fig 4) All of these excitatory responses occurred after a latency of 2—3 sec, and the optimal effect was reached at a stimulus frequency of about 50 cps

Inhibitory stimulation effects were recorded as a diminution or abolition of the rhythmic bladder activity (Fig 5) arrest of micturition (Fig 6 and Fig 7b) or prevention of the onset of urination (Fig 7c—d) Arrest of micturition could be demonstrated irrespective of the urination reflex was initiated by cortical stimulation or resulted from bladder distention A latency of 2—3 sec was found also in all types of inhibitory bladder response this being recorded as a prompt and marked reduction of the intravesical pressure A drop of tension to below the resting level (as recorded in the intervals between two successive spontaneous detrusor contractions) was often encountered On cessation of stimulation a sharp pressure rise was frequently observed with restoration of the pre stimulation tension level In some cases however a post stimulation inhibition of long duration (2—3 min) was obtained

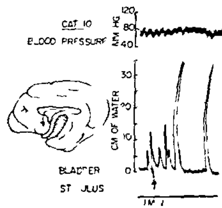


Fig 6 Curarized cat Interruption of urination initiated by rapid bladder filling by stimulation (5 volts 50 cps) of the point indicated by [illegible] in anterior cingulate region The inhibitory effect was succeeded by an immediate intravesical pressure rise and re-occurrence of micturition on cessation of stimulation

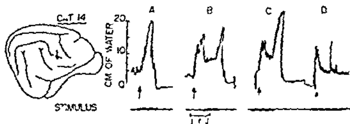


Fig 7 Interruption (*B*) and inhibition (*C D*) of normal micturition act (*A*) initiated by rapid bladder filling These inhibitory responses were obtained from the area indicated by arrow on the inset. The duration of the inhibition corresponds to the stimulation period Stimulus parameters 1 msec 7 volt 50 cps The effects shown in C and D were produced when stimulation was started 15 sec prior to the bladder filling

Suppression of the urination mechanism could be produced when cortical stimulation was started prior to a rapid bladder filling beyond the volume which would ordinarily release the micturition reflex in that particular experiment In Fig 7c the stimulus was applied to an inhibitory point of the cerebral cortex 15 sec before the filling of the bladder A delay of urination is shown, lasting until cessation of the cortical stimulation As illustrated in Fig 7d the voiding act could also be completely inhibited Inhibitory bladder responses were obtained by the same stimulation parameters which gave optimal excitatory effects They were however elicited within a wider frequency limit from 5 to 50 cps being maximal between 20 and 50 cps

During exploration of the responsive cortical regions the same type of bladder reaction could be readily and repeatedly reproduced from each point of stimulation In the same animal opposite stimulation effects were however regularly observed on shifting the site of the electrodes

The rhythmic bladder activity as well as any effect of cortical stimulation were depressed by deepening the anesthesia

The cortical origin of the excitatory and inhibitory bladder responses was demonstrated by the fact that local application of 1 per cent lidocaine (Xylocain® Astra) to the cerebral cortex abolished the effects whereas the rhythmic activity of the bladder was not interfered with

The genuineness of the bladder responses One might question whether the hydrostatic pressure changes recorded from the vesical cavity did in fact reflect exclusively variations in bladder activity Several factors which might be assumed to influence the dynamics of the bladder have to be considered

Firstly the possibility that the intravesical pressure changes are secondary to alterations in respiratory movements is excluded by the demonstration of their presence in the curarized and artificially ventilated animal As shown in Fig 4 and 6 the rhythmic detrusor contractions were not altered by giving paralyzing doses of succinylcholine marked stimulation effects excitatory (Fig 4) as well as inhibitory (Fig 6) were readily elicited

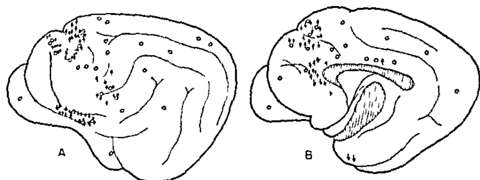


Fig 8 Bladder responses to stimulation of the cerebral cortex in cats A Lateral aspect of the cerebral hemisphere B medial aspect Excitation ↑ inhibition ↓ and no response o

Secondly, contractions of the abdominal muscles may effect the intra abdominal pressure and thereby might be assumed to influence the bladder activity However the presence of bladder responses in fully curarized animals also proves them to be independent of this factor Further definite bladder responses were frequently observed without any visible movements of the abdominal wall

The probability remains that alterations in gastrointestinal movements may act directly upon the bladder wall and in this way may interfere with the stimulation effects This is strongly contradicted by the fact that forceful ileus like bowel contractions (visible by external abdominal inspection) resulting from stimulation of the vagus nerve did not affect the intravesical pressure as recorded on the polygraph

Localization of the responsive cortical regions The topographical distribution of the cortical points which have been stimulated in the present experiments appear on Fig 8 No change in the bladder activity resulted from stimulation of extensive areas on the lateral and medial aspects of the hemispheres and the olfactory bulb Most of the temporal lobe including the hippocampus was not explored This will be done in a subsequent study which also deals with the effects of stimulation of the amygdaloid and periamygdaloid region Significant stimulation effects on the bladder activity were obtained from the following cortical regions

1 The first somatic sensory motor cortex This region was explored in 12 animals in which a total of 60 stimulations were applied 28 excitatory and 19 inhibitory responses were obtained whereas 13 stimulations produced no bladder response Both types of bladder reaction were encountered in all the 12 cats although at times excitatory or inhibitory stimulation effects predominated On the lateral aspect of the hemisphere the responsive loci occupy the upper portion of the pericruciate cortex and the anterior as well as the posterior sigmoid gyri The

effective zone seems to include the region of the lower extremities and trunk whereas the region of the upper extremities and the head is unrespon ive On the medial aspect of the hemisphere excitatory effects were mainly obtained from the anterior sigmoid gyrus whereas inhibitory reactions predominated when stimulating the posterior sigmoid gyrus

2 *The anterior ectosyl ian and anterior sylvian gyri corresponding to the somatic sensory motor area II as outlined by Woolsey et al* (1958 1959) This region like the perncruciate cortex yielded both types of bladder response However in 2 of the 5 animals in which this cortical region was explored inhibitory effects only were recorded Excitatory as well as inhibitory responses were produced in the remaining 3 cats As a whole the stimulation effects were not as readily obtained from this area as from the first somatic sensory motor cortex They seemed to depend more on the animal being in good condition and on a relatively light anesthesia

3 *The anterior cingulate cortex* (Fig 8b) The different character of the bladder response allows a clear subdivision of this area into two zones one lying superiorly and bordering the genus of the corpus callosum the other lying inferiorly in the subcallosal part of the cingulate gyrus Stimulation of the supracallosal and pregeniculate cortex was performed in 8 animals consistently resulting in bladder excitation In 4 of the same cats the subcallosal part of the anterior cingulate region was exposed All of them responded to stimulation of subcallosal points giving inhibitory bladder effects exclusively

4 *The orbital gyrus* (Fig 8a) The bladder reactions produced by stimulation of this region were consistently of the inhibitory type Such effects were obtained in 7 animals in which a total of 28 stimuli were applied Four of these stimulations caused no change in the bladder activity whereas inhibition occurred 24 times

(C) *Concomitant changes in blood pressure and respiratory mo ements*

In accordance with previous observations (for references see KAADA 1951 1960 UVNAS 1960 and LOFVING 1961) alterations in blood pressure and respiratory movements were recorded by stimulation of all the cortical regions found to yield bladder responses

Effects on blood pressure The pressure alterations as recorded from the femoral artery, were in general slight and inconstant This fact may probably be related to the type and depth of anesthesia used in the present study No definite correlation was shown to exist between the direction of the pressure alteration in the femoral artery and in the vesical cavity The diagrams in Fig) and (serve to illustrate this independence by showing two ca es of bladder inhibition one being accompanied by a blood pressure fall and the other by a slight elevation

Effects on respiratory mo ements In general the respiratory responses were the same as those reported previously As a rule these stimulation effects were more marked and more readily obtained than the blood pressure changes The

most striking influence on the respiratory movements was elicited from the orbital and anterior cingulate regions from which an initial complete arrest in expiration was produced (Fig 3a) usually followed by a period of lowered respiratory frequency The somatic sensory motor areas I and II yielded weaker respiratory responses but acceleration as well as slowing of the respiratory rate were frequently observed

Discussion

The present experiments demonstrate that during chloralose urethane or nembutal anesthesia rhythmic bladder contractions can be recorded in the cat The frequency and amplitude of the contractions are augmented by increasing bladder distention which finally results in urination

Previous cortical stimulation experiments have chiefly been concerned with the influence on the rhythmic bladder activity The recording technique used in the present investigation allows in addition a comparison of the influence on the rhythmic detrusor contractions and the micturition reflex All the responsive areas were shown to produce either of these effects both as regards excitation and inhibition However the latter was often significant only when demonstrated by the interruption of micturition

The following *excitatory* stimulation effects have been demonstrated (i) augmentation of the rhythmic detrusor activity (ii) the initiation of repetitive and strong contractions resulting in dribbling urination and finally (iii) a sustained detrusor activity representing the normal micturition act with full emptying of the bladder The present investigation confirms the earlier experimental evidence of an excitatory influence on the bladder activity from the first somatic sensory motor cortex and the supracallosal part of the anterior cingulate region In addition excitatory (as well as inhibitory) bladder responses have been elicited from the anterior ectosylvian and anterior sylvian gyri corresponding to the somatic sensory motor area II as delineated by WOOLSEY *et al* (1958 1959) This is to the authors knowledge the first demonstration of an autonomic response to stimulation of this area

In accordance with previous observations (LANGWORTHY and KOLB 1935 SPIEGEL and HUNSICKER 1936) *inhibitory* bladder reactions were demonstrated by cortical stimulation of the first somatic sensory motor cortex Further bladder inhibition was in the present study elicited also from the somatic sensory motor area II from the subcallosal part of the anterior cingulate gyrus and from the orbital region The two latter regions yielded inhibitory responses exclusively and the effects were strong and readily obtained especially as far as the influence on the micturition act was concerned Thus within the anterior cingulate area there appear to be two distinct zones one exerting an excitatory and one an inhibitory influence on the bladder activity A similar differentiation has been made by KAADA (1951 1960) with regard

to the effects on somatomotor activities and partly on respiration and blood pressure Further it is of considerable interest that in the cat bilateral ablation of the same two zones results in different behavioral manifestations (McCleary 1960 Kaada Kveim and Rasmussen 1962) In contradiction to the results of the present investigation Koikegami *et al* (1957) reported no inhibitory effects on the bladder activity by stimulation of the orbital gyrus This discrepancy may be due to differences in the experimental techniques The latter investigators recorded the intravesical pressure either by the use of an intravesical balloon or by establishing a closed cavity by means of a catheter Thus the effects on the micturition act, which appears to be of special importance in studies of the inhibitory influence could not be observed Bladder inhibition was also produced by stimulation of the pyriform cortex through needle electrodes Such stimulation was carried out in two animals in addition to the usual experimental procedure (Fig 5)

The short latency of the bladder responses proves that they are mediated by nervous activity and not through a humoral mechanism The cortical origin of the effects is demonstrated by their disappearance on surface anesthesia of the responsive cortical locus A study of the pathways mediating the impulses from the responsive cortical regions will be dealt with in a subsequent communication

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Acta physiol scand 1963 59 349—356

From the Institute of Physiology University of Uppsala Sweden

A Method for Continuous Monitoring of the Rapid Uptake of Ions by Biological Tissues

By

B DANIELSON and U SJOSTRAND

Rec ed 7 March 1963

Abstract

DANIELSON B and U SJOSTRAND *A method for continuous monitoring of the rapid uptake of ions by biological tissues* Acta physiol scand 1963 59 349—356 — A method is described with which the uptake of ions by biological tissues can be studied continuously The method makes possible studies during short time intervals (0 1 sec) The preparation is mounted in a chamber which is inserted in a gamma ray analyzer Through the chamber is flushed Ringer s solution interrupted by periods of silicone oil The silicone oil is inert to the system The preparation used in this paper was the sinus venosus of the frog s heart Influx curves for K^{42} and Na^{24} are presented

It is known that there are different ionic concentrations (e g K^+ Na Cl^- and HCO_3) in the intracellular and the extracellular spaces due to metabolic and other conditions

The resting potential of the cell membrane has been regarded as the result of the ionic distribution and fluxes during the resting state Changes in this distribution have been proposed as responsible for the reversal of the polarity of the cell membrane that occurs during an action potential in excitable cell (HODGKIN and HUXLEY 1952 a—d) This would mean that the ionic fluxes were also changed during activity

Many attempts have been made to clarify experimentally the relations between ionic fluxes and electrical activity Both influx and outflux studies have been performed and in most of the experiments radioactive isotopes have been used The fluxes have been examined during long time periods (> 15 min) and under different conditions (resting state and varied temperatures ionic conditions and stimulation frequency etc) in e g nerve and heart tissue

results quantitative calculations regarding the total ionic fluxes expected during one single action cycle have been made

The *outflow* experiments have as a rule been performed as follows A tissue preparation has been loaded with the radioactive isotope to be studied After a sufficient loading time the preparation is soaked in inactive bathing solution, which is then analyzed for radioactive tracer (c f among others JOHNSON 1957 RAYNER and WEATHERALL 1959)

In a few papers outflux studies during single action cycles were reported WILDE and O BRIEN (1953) studied the K^{42} concentration of coronary perfusate from an isolated turtle heart in relation to the recorded ECG They found periodic changes in K^{42} activity with a peak during the T wave of the ECG DANIELSON, ÖBRINK and SJÖSTRAND (1959 1961 1962) perfused inverted atrial and sinus venosus preparations which were loaded with Na^{24} and K^{42} The Na^{24}-concentration of the perfusate varied synchronously with the electrical activity An efflux peak regularly appeared during the last part of the repolarisation The K^{42} concentration also showed variations in phase with the electrical cycle LORBER *et al* (1962) reported similar results for the K^{42} efflux from a ventricular strip They found a well defined efflux peak that regularly occurred *synchronously with and was of approximately the same duration as the action* potential

These experiments however only give information about the tracer flux in one direction i e from the preparation to the perfusate The only conclusion that can be drawn is that there are periodic variations in the tracer fluxes In order to study the complete pattern of the ionic fluxes it is necessary to study the tracer fluxes in both directions

Ionic *influx* measurements have been reported previously The method most used for studying the inflow of ions into a tissue preparation has been to measure the radioactivity of the preparation after soaking for different times in a solution containing the radioactive isotope (see e g JOHNSON 1957) Such experiments have been performed on different tissues and under different conditions From integrated influx experiments of this type it is impossible however to draw any conclusions regarding the ionic influx during a single cardiac cycle To be able to follow the tracer flux into the preparation during the cardiac cycle it is necessary to use a method which permits continuous recording of the radioactivity of the preparation

Hitherto studies following continuously the uptake of ions do not seem to have been reported A method for such uptake measurements is described in this paper A preliminary report has been published earlier (SJÖSTRAND 1962)

Principle of the fractioned uptake method

The purpose of inflow measurements is to follow the tracer uptake by a preparation from the surrounding solution by analyzing the tracer content of the preparation after different times A necessary condition for such a procedure

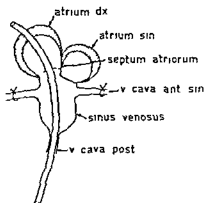


Fig 1 The isolated sino-atrial preparation The position of the plastic catheter after its introduction through the atrioventricular opening is shown The dashed line between the sinus venosus and the atria represents the sulcus circularis = section line (cf Heart preparation)

is that the surrounding radioactivity is small compared to the radioactivity of the preparation itself during the time when the radioactivity is being measured If the tracer influx is to be studied during short intervals e g during the single heart cycle it is particularly difficult to reduce the background activity during the determination of the radioactivity

In analyzing the tracer uptake of the sinus venosus this was perfused intermittently with a radioactive solution The perfusion was interrupted by a flow of silicone oil of low viscosity The oil flushes away the radioactive solution that surrounds the preparation During this time the radioactivity of the preparation can be determined

Methods

Heart preparation

The experiments were performed on the isolated sinus venosus of the frog (*Rana temporaria*) or the toad (*Bufo bufo*) Frog or toad heart was chosen because it has no coronary system and the normal mode of nutrition is by a direct transport of fluid and solutes between the lumen of the heart and the spongy structure of the walls This means that perfusion of the endothelial side of the sinus venosus simulates the normal mode of nutrition of the cells The preparation had a spontaneous rhythmicity (primary and potential pace makers)

After decapitation of the frog the heart was dissected free and the sinus venosus cleared of surrounding connective tissues For anatomical details c f ECKER and WIEDERSHEIM (1904 p 269) Both anterior caval veins were ligated as far distal from their entrance into the sinus venosus as possible The sinus venosus was cut away from the atria along the sulcus circularis A plastic catheter was introduced through the sino-atrial opening and further into the posterior caval vein and through the wall of this vessel (see Fig 1) The posterior caval vein was then cut and the preparation freed from the frog The sinus venosus was then transferred from the plastic catheter on to another catheter which was fixed in the U tube shown in Fig 2 The sinus venosus was then turned inside out (endothelial side = outside) and was tied to the catheter at both ends (Fig 3) The endothelial side of the sinus could now be irrigated (superfused) without

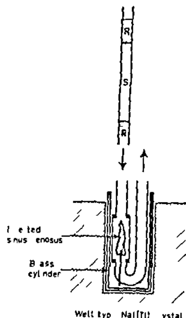


Fig 2 The isolated inverted sinus venosus preparation of Fig 1 in position in the U tube used for superfusion (Sjöstrand 196?) The superfusing chamber is mounted in the well of the NaI(Tl) crystal The alternate flow of the Ringer s solution (R) and the silicone oil (S) is schematically shown

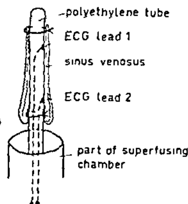


Fig 3 The isolated inverted sinus venosus preparation Two silver wire leads 1 and 2 on the epicardial si l are shown

distension of the preparation An increase of the tension of the wall of the sinus venosus is known to increase the spontaneous activity of the preparation (PATHAK 1958 MARWOOD 1962)

Technical arrangements

The inverted sinus venosus was mounted in a chamber of relatively small dimension see Fig 2 The perfusion chamber was inserted in a brass cylinder which in turn lay in a NaI(Tl) well type crystal The brass cylinder was thick enough to absorb all beta rays with energies lower than 3 5 MeV The sinus venosus could now be perfused with Ringer s solution for short time intervals interrupted by periods of low viscosity silicone oil (5 cS) The silicone oil was obtained from Midland Silicones Ltd London England

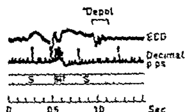


Fig 4 Part of a recording from an inflow experiment on an isolated inverted sinus venosus (from Sjostrand 1963) The propagation of the depolarisation is indicated by Depol The perfusing situation is schematically indicated above the time scale 10- and 100-p ps are shown on the decimal pip registration

The length of the periods could be varied from about 0 1 up to several seconds A radioactive isotope Na^{24} or K^{42} was added to the Ringer s solution If the perfusion rate was high enough turbulent flow and sharp phase boundaries between silicone and aqueous regions were obtained A detailed study will be published later by one of us (SJOSTRAND 1963)

In the experiments the perfusion temperature was + 20 C At this temperature the frequency of the *sinus venosus* was 25—30 beats per minute The heart frequency was determined from an ECG recording (two leads on the epicardial surface of the preparation see Fig 3)

The silicone oil flushed away nearly all of the radioactive Ringer s solution surrounding the preparation The Na^{24} or K^{42} activity (mainly within the preparation) could be analyzed during the silicone perfusing period The intermittent flow of the Ringer s solution and the silicone oil was obtained by a rapidly working two-way valve giving sharp phase boundaries

In order to register the radioactivity of the preparation during the silicone oil period without interference of the surrounding high activity periods a rapidly working recording system was necessary with time constant practically zero The recording system used was as follows All pulses from the analyzer were registered by an ordinary scaler Every 10 100 and 1 000 impulses from the scaler were recorded with a decimal pip method (DANIELSON *et al* 1963) on an oscilloscope screen which was photographed continuously Generally a sufficient number of pulses were recorded to give an accuracy of 1—5 % (DANIELSON and SJOSTRAND 1963) The ECG was fed to a second channel of the oscilloscope (Tektronix mod 561) see Fig 4

The perfusion chamber was mounted in an 1 3/4 NaI(Tl) well type crystal in combination with RCA 6655 photomultiplier (Tracerlab RLD — 3) The high voltage was obtained from a Tracerlab Precision High Voltage Supply with a long time stability of 0 02 % per day Linear amplifier and pulse height analyzer were Tracerlab RLI — 4

Solutions

Normal Ringer s solution

6 5 g NaCl (111 2 mM 0 2 g KCl (2 68 mM) 0 2 g $CaCl_2$ (1 80 mM) 0 1 g $NaHCO_3$ (1 19 mM) and 1 g glucose (5 55 mM) per liter H_2O (pH = 7 2)

In experiments where Na^{24} inflow was studied 0 03 g Na^{24} Cl was added to 0 5 l normal Ringer s solution The specific activity for the perfusing solution was 11 mC per g Na In experiments with K^{42} 0 43 g K^{42}Cl was added to 0 5 l normal Ringer s solution The resulting specific activity was calculated as 38 2 mC per g K Na^{24} and K^{42} were obtained by irradiation of $NaHCO_3$ and K_2CO_3 respectively

The isotopes were obtained from AB Atomenergi Stockholm Sweden

Table I The Ringer s solution contained 37 200 ± 427 cpm per ml

Sample	Mixing time in minutes	Sample activity — background in cpm	Coefficient of variation in per cent
1	10	26	56 2
2	20	15	98 3
3	60	13	118 0
4	120	30	50 0
5	200	8	197 0
6	300	33	46 0

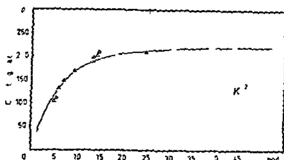


Fig 5 Inflow experiments for K Abscissa Accumulated perfusing time with K^{42} Ringer s solution Ordinate Counting rate (cps) during the silicone oil periods succeeding each Ringer period Duration of the whole experiment (Ringer and silicone periods) 210 seconds

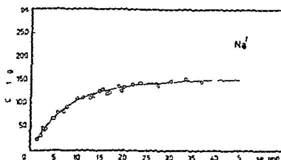


Fig 6 Inflow experiment for Na (the same preparation as in Fig 5) Abscissa Accumulated perfusing time with Na Ringer s solution Ordinate Counting rate (cps) during the silicone oil periods succeeding each Ringer period Duration of the whole experiment (Ringer and silicone periods) 210 seconds

Ionic distribution between Ringer s solution and silicone oil

It was of interest to examine the distribution of ions between Ringer s solution and silicone oil This was done in experiments where equal volumes of Ringer s solution containing Na^{24} and K^{42} respectively and silicone oil were shaken together Samples from the silicone oil were taken after shaking for 10 20 60 120 200 and 300 min Table I shows the result

Table I shows that no radioactive ions were detected in the silicone oil A similar result was obtained for K^{42} where the Ringer s solution contained 15 785 ± 174 cpm per ml The radioactivity of a silicone oil sample after 400 min mixing time was not significantly different from the background level Thus Na^{24} and K^{42} did not dissolve in the silicone oil used

Results

As the aim of this paper is to present a method which makes possible intermittent monitoring of a tracer influx in a preparation the presentation of fuller details concerning the cardiac cycle will be deferred to a later publication Fig 5 shows an inflow experiment for K^{42} in an isolated spontaneously beating sinus venosus of the frog The accumulated perfusing time with K^{42} Ringer s solution is plotted on the abscissa and on the ordinate the counting rate during the silicone oil period succeeding each Ringer period

Fig 6 shows corresponding data for Na^{24} in the same preparation a few minutes later This was made possible by raising the discrimination level of the pulse height analyzer above the photopeak of K^{42}

Discussion

The fractionated uptake method reported makes it possible to study ionic uptake in tissues continuously and to detect rapid changes in the uptake rate Perfusing systems with radioactive Ringer s solution interspersed with analyzing periods of air or inactive Ringer s solution was found to be insufficient Silicone oil of viscosity nearly equal to that of the Ringer s solution offers many advantages It is strongly hydrophobic and electrolytes do not dissolve in it Additionally it seems biologically inert and apparently does not affect the heart tissue This was shown in experiments where the isolated spontaneously beating sinus venosus was taken out of the bathing Ringer s solution and put into silicone oil

Figs 5 and 6 show that almost stable levels of the counting rates for both K^{42} and Na^{24} were reached within a short time Earlier experiments (e g Johnson 1957) showed a longer time to reach the plateau level but they were performed on frog ventricle which with its thick wall makes direct comparison between the two types of experiments impossible

As pointed out in the introduction it is supposed that ionic fluxes occur during the electrical activity of excitable tissues according to the most accepted hypotheses Only a few investigations are published which show such ionic fluxes during one single action This paper describes a method which may make possible studies of the influx of ions into biological tissue A primary condition for studies of exit and entrance during electrical activity in excitable tissues is that the preparation can be analyzed at very short time intervals From a technical point of view the method described satisfies this condition

Our sincere thanks are due to Mrs M Waller for technical assistance and Miss I Sjöstrand for preparing the manuscript This investigation was supported by a PHS research grant H 5346 from the National Heart Institute Public Health Service USA and grants from the Medical Faculty University of Uppsala the Swedish National Association against Heart and Chest Diseases Stockholm and the Swedish Society for Medical Research Stockholm

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Acta physiol scand 1963 59 357—362

Aus der Klinisch physiologischen Abteilung des Karolinska Sjukhuset Stockholm
Schweden

Rontgenologische Bestimmung des Herzvolumens der Ratte

Von

Bernhard Tribukait

Eingegangen am 9 Marz 1963

Abstract

Tribukait B *Rontgenologische Bestimmung des Herzvolumens der Ratte* Acta physiol scand 1963 *59* 357—362 — A method for X ray determination of the heart volume of rats is described Using general anesthesia the heart was X rayed in the frontal and sagittal planes The relative volume of the heart calculated from the length breadth and depth was multiplied by the correction factor of 0 44 to obtain the absolute volume The correction factor has been estimated by empirical analysis in heart models The relationship between length (a) breadth (b) and the square of depth (c) i e $\frac{c}{a \times b}$ does not change during growth The standard error of estimate for a single determination calculated from 168 duplicated determinations was 7 3 %

Systematische Untersuchungen des menschlichen Herzvolumens haben vielfach wesentlich zur Beurteilung des pathologischen und zum Verstandnis des physiologischen Kreislaufgeschehens beigetragen Derartige Studien scheinen bei Tieren nicht vorzuliegen

Um Kenntnisse uber das Verhalten des Herzvolumens der Ratte und dessen Stellung innerhalb des Kreislaufs unter verschiedenen experimentellen Bedingungen zu erhalten ist eine Methode zur rontgenologischen Herzvolumenbestimmung ausgearbeitet worden die nachfolgend beschrieben werden soll

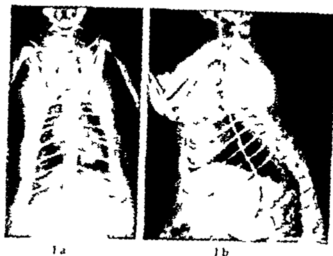


Abb 1 Frontaler und seitlicher Herzschatten einer Ratte

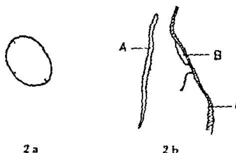


Abb 2 Schematische Wiedergabe des frontalen und seitlichen Herzschattens mit Längs Quer und Tiefendurchmesser A Brustbein B Trachea C – Ösophagus

Methodik

Vom Herzen der Ratte werden in Bauchlage und linker Seitenlage Röntgenbilder hergestellt Um das Versuchstier in der gewünschten Lage zu halten ist eine Narkose notwendig (Basisnarkose mit Nembutal 1 2 mg/100 g Körpergewicht i p Äthernarkose bis zur Entspannung) Durch einen Katheter wird in den Ösophagus etwas Röntgenkontrastbrei eingespritzt Wie bei der Herzvolumenbestimmung an liegenden Menschen (KJELLBERG RUDHE und SJÖSTRAND 1949) wird um die Herzkonturen klarer herauszuprojizieren bei der Frontalaufnahme der Zentralstrahl um einen Winkel von 30 caudalwärts gerichtet das Seitenbild wird mit perpendikularen Strahlen angefertigt Der Abstand Röntgenröhre—Röntgenplatte beträgt 120 cm Belichtungszeit 3 5 mAs bei 40—50 kV und 700 mA (– 0 05 sec) Die so erhaltenen Herzschatten zeigen Abb 1 a und 1 b

Der frontale Herzschatten wird der Länge und der Breite der seitliche Herzschatten der Tiefe nach ausgemessen Dazu wird die Herzspitze mit der äusseren Begrenzung des rechten Vorhofes verbunden senkrecht zu diesem

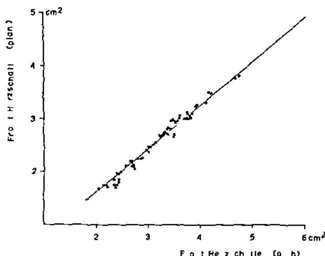


Abb 3 Beziehung zwischen dem planimetrisch gemessenen frontalen Herzschatten (Ordinate) zu dem Produkt aus Herzlänge (a) und Herzbreite (b) (Abszisse) Gleichung der Regressionslinie y = 0 82 x — 0 01 r = 0 96 Werte von 119 Tieren

Längsdurchmesser und durch dessen Mitte verläuft der Querdurchmesser (Abb 2 a) Zur Tiefenbestimmung des Herzens ist der Abstand Innenrand des Sternums bis zu der gut zu erkennenden Einmündung der Lungengefässe in den Herzschatten gewählt wobei der mit Kontrastbrei gezeichnete Ösophagus die genaue Lokalisation erleichtert (Abb 2 b) Die Abstände lassen sich auf 1/2 mm genau bestimmen

Aus diesen drei Grössen wird das Produkt gebildet und dadurch ein relatives Mass für das Herzvolumen[1] erhalten das indessen die Form eines Würfels oder Quaders hat nicht aber die eines angenäherten Rotationsellipsoids wie es das Herz darstellt Um von diesem relativen zum absoluten Herzvolumen zu gelangen wird das relative Herzvolumen mit einem Korrekturfaktor von 0 44 multipliziert der in der folgenden Weise ermittelt worden ist Von den Herzen zweier unterschiedlich grossen Ratten wurden mit Hilfe von Gipsabdrücken Phantomherzen aus Blei hergestellt deren Absolutvolumina unter Anwendung des Archimedes schen Prinzips gemessen wurden Diese Phantomherzen wurden dann getöteten Tieren eingesetzt und deren relative Volumina analog den lebenden Herzen aus den angefertigten frontalen und seitlichen Röntgenabbildungen bestimmt Die erhaltenen Umrechnungsfaktoren betrugen 0 45 und 0 43

Der Methodenfehler der an Hand von 168 Doppelbestimmungen bei 100—500 g schweren männlichen Ratten eines Stammes (Wistar) ermittelt wurde beträgt für eine Einzelbestimmung 7 3 %

[1] Unter relativem Herzvolumen wi d in d r röntgenologische i Fach ; rache das Herz olumen/m Körperoberfl che erstar den

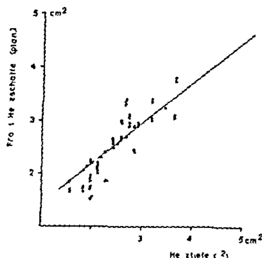


Abb. 4. Beziehung zwischen dem planimetrischen gemessenen frontalen Herzschatten (Ordinate) zu dem Quadrat der Herztiefe (c^2) (Abszisse). Gleichung der Regressionslinie $y = 0{,}78\,x + 0{,}$[illegible] $r = 0{,}80$. Werte von 119 Tieren

Diskussion

Auf die Prinzipien der röntgenologischen Volumenbestimmung eines unregelmässigen Körpers, wie ihn das Herz darstellt, ist im Zusammenhang mit der menschlichen Herzvolumenbestimmung von LARSSON und KJELLBERG (1948) ausführlicher eingegangen worden. Exakte Volumenbestimmungen verlangen eine grössere Zahl von Projektionen in verschiedenen Ebenen. Aus praktischen Gründen muss man sich jedoch auf einige wenige Messgrössen beschränken, mit deren Hilfe dennoch das Herzvolumen möglichst genau berechnet werden soll.

Bei der vorliegenden Methode wurde das Herz frontal und seitlich röntgenphotographiert. Es erschien am einfachsten und ist auch berechtigt, den frontalen Herzschatten nur der Länge und Breite nach zu messen. Wie Abb. 3 zeigt, steht das aus diesen beiden Grössen berechnete Rechteck in einer engen Beziehung zur planimetrisch ausgemessenen Fläche.

Der zum Errechnen des absoluten Herzvolumens empirisch ermittelte Korrektionsfaktor trägt praktisch nur der Form Rechnung, da bei den fast parallel einfallenden Röntgenstrahlen die Vergrösserung äusserst unbedeutend ist. Der gefundene Zahlenwert hat allerdings nur dann allgemeine Gültigkeit, wenn die Form (oder auch Lage) des Herzens unverändert bleibt. Beim Menschen ist das nicht der Fall. Zwischen der Grösse des frontalen Herzschattens und der Tiefe als Mass für die Form des Herzens fand LUDWIG (1939) keine Korrelation. LARSSON und KJELLBERG (1948) haben aus diesem Grund verschiedene Zahlenwerte des Korrektionsfaktors berechnet. Die anatomischen Verhältnisse der Ratte sind dagegen sehr viel konstanter. Dies geht aus Abb. 4 hervor, in der von 119 verschiedenen 100—500 g schweren Ratten die Fläche des frontalen Herzschattens auf der Ordinate gegen das Quadrat der Herztiefe auf

Tab I Her form J der Ratte berechnet aus dem Verhaltnis zwischer dem Qu adrat d T fe (c) und dem Produkt aus Lange (a) und Breite (b)

$J = \frac{c}{a \times b}$ Mittelwerte von Gruppen verschiedener Herzgrossen.

Zahl	Herzgrosse (ml)	J
30	< 2 00	0 800 ± 0 019
30	2 01–3 00	0 819 ± 0 019
30	> 3 01	0 811 ± 0 072

der Abszisse aufgetragen worden ist Mit grosserer Herzlange (a) und Breite (b) wachst auch die Tiefe (c) Bestimmt man den Quotienten J aus dem Quadrat der Tiefe und dem Produkt aus Lange und Breite $\left(J = \frac{c^2}{a \times b}\right)$ wie es in Tab I für Gruppen verschiedener Herzgrossen geschehen ist findet man keine statistisch zu sichernden Unterschiede ($p > 0\,05$) Es ist deshalb zumindest unter Normalbedingungen moglich (wenn auch im Hinblick auf die Genauig keit der Herzvolumenbestimmung nicht empfehlenswert) lediglich aus dem frontalen Herzschatten der Ratte auf das Herzvolumen zu schliessen wie es STICKNEY NORTHRUP und VAN LIERE (1956) getan haben

Ob die kurz dauernde Narkose auf die Grosse des Herzvolumens einen Ein flu s hat konnte nicht geklart werden Selbst zahme Tiere waren ohne Narkose nicht in der gewunschten Stellung zu halten Da Narkotika die Blutverteilung verandern konnen (RIEKE und Everett 1957 — Ratte FRIEDMAN 1959 — Maus VIDT *et al* 1959 — Ratte) ist eine Ruckwirkung auf das Herzvolumen denkbar In wie weit dabei eventuelle Variationen des Herzvolumens mit Änderungen der Pulsfrequenz oder des Blutdrucks zusammenfallen muss spe ziellen Untersuchungen vorbehalten bleiben

Einen bestimmten Abschnitt der Herzphase zu erfassen wurde aus prak tischen Grunden und der Überlegung heraus dass die Volumenveranderungen der Ventrikel zumindest zum Teil durch entgegengesetzte Volumenverande rung der Vorhofe kompensiert werden nicht versucht Ebenfalls aus praktischen Grunden wurde die Atemphase mit ihren moglichen Einwirkungen auf das Herzvolumen unberucksichtigt gelassen

Mi t Unterstutzung Prof T SJÖSTRAND V r fugung gestellt n M tteln des R ksidrottsfor bundets Pol kl nikkomm ttd

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Acta physiol scand 1963 59 363—369

From the Institute of Zoophysiology University of Uppsala Sweden

The Influence of Oxytocin upon Some Seminal Characteristics in the Rabbit

By

J E Kihlström and P Melin

Received 13 March 1963

Abstract

Kihlström J E and P Melin *The influence of oxytocin upon some seminal characteristics in the rabbit* Acta physiol scand 1963 59 363—369 — Seminal volume sperm count and sperm density were studied in ejaculates collected from rabbits immediately after intravenous injections of oxytocin The hormone was shown to increase the volume of the first ejaculate obtained and incite a more rapid emission of sperm cells in the ejaculates immediately following Some observa tions may indicate that the oxytocic effect varies with the androgenic status of the animal Results have been presented suggesting a release of gonadotropins caused by oxytocin

The effects of oxytocin on the female sex organs are well known Furthermore it has been established that this hormone is released by uterine distension by coitus and by suckling and its role in parturition has been discussed However the effects of oxytocin upon male functions are less studied (Waddell 1916 1917 Perutz and Merdler 1924 Laquer de Jongh and Tausk 1948 Cross 1955 1959 Cross and Glover 1958 Dziuk and Norton 1962 Ewy and Bielanski 1962) Lately Debackere Peeters and Tuyttens (1961) have shown that upon stimulation of the genitals also in males an oxytocic substance is liberated from the posterior lobe of the pituitary It thus appears as if oxytocin could have a direct and rapid effect in connection with male sexual functions We therefore studied the density of sperm cell and volumes of ejaculates produced immediately after an oxytocin injection Besides the effects of this hormone upon the libido and a delayed action on sperm count were observed, and will be reported in separate papers (Melin 1963 Melin and Kihlström 1963)

Recently cyclic variations in the sexual functions of the male rabbit have been demonstrated (Doggett 1956 Kihlström 1958 Degerman and Kihl-

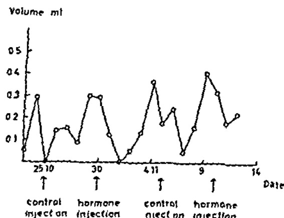


Fig 1 Variation in semen volume of the first ejaculate in one rabbit and the distribution of some of the injections

STROM 1961) These phenomena are caused most likely by cyclic variations in the androgenic status of the animals (KIHLSTROM and DEGERMAN 1962) In females the effects of oxytocin are influenced quantitatively and qualitatively by other sex hormones We therefore also studied whether or not oxytocin causes different effects in different phases of the male sexual cycle

Material and Methods

Nine male rabbits of the Swedish Country Breed have been studied from Sept 15 1961 to April 14 1962 The animals were all sexually mature and none of them more than two years old The bucks were kept separately and in the same cages throughout the experimental period and were given the same kind of food once a day at a given time The experiments and the daily sperm collections were performed at 2 p m

The hormone preparation was a purified posterior lobe extract (Utedrin Astra Ltd the ratio of its oxytocic and pressor activities being 100/3 The doses were in 3 animals 0 15 I U in 5 animals 0 30 I U and in one animal 0 60 I U per kg body weight A physiological sodium chloride solution was used for control injections Each animal served as its own control All injections were done in the marginal ear vein

With a doe present in the cage the animals were accustomed by training to ejaculate into an artificial vagina Does have been used as sexual stimuli instead of rabbit furs since some bucks exhibited no sexual interest in a fur The experiments were then performed in the following way 30 sec after the Utedrin injection a doe was placed in the cage of the male animal The buck could then ejaculate repeatedly during 10 min or until exhausted (= exhaustion experiment) As it could be suspected that the bucks reacted differently to different does the same doe was used for each buck When the does were in oestrus apart from the daily semen collecting no experiments were performed

The semen was collected in small test tubes the volumes of which up to a mark in the glass wall were known with great accuracy By adding a ? per cent water solution of chloramine which effectively kills the sperm cells from a burette up to this mark the volume of the semen could be determined with a high degree of precision (DEGERMAN and KIHLSTROM 1961 About half the number of the ejaculates contained gelatinous mass which were removed before the determination of the volume According to the density the semen was diluted 50 to 200 times and a small portion was transferred to a hemocytometer After 10 min when the sperm cells had settled down the sperm density was

determined under a microscope by counting at least 200 cells Some ejaculates contained urine that was easily observed by its colour and odour Such ejaculates particularly frequent in one animal have been excluded

The male sexual cycle was determined in each animal by observing the daily variations in the volumes of the ejaculates Besides during a fortnight before the start of the experiments the bucks were given daily saline injections in connection with semen collections By this treatment the animals became familiar with the experimental procedures

In 4 animals the hormone was injected the day after a peak in the semen volume and in 5 individuals the injections were performed the day after a minimum volume Each animal was given between 3 and 5 hormone injections and with a few exceptions the same number of control injections Fig 1 shows the variation in semen volume of the first ejaculate of one animal and also the distribution of some of the injections In a few cases when the hormone had been given the day after what was regarded as a peak-volume the animals yielded a still higher volume It is impossible to decide whether this was due to the injected hormone or the fact that the injection had not been given after maximum We have therefore excluded these experimental data

In the experiments the first ejaculate and those immediately following are of course the most interesting However in many cases the volumes of one or more ejaculates were too small to be measured In such cases the volume has been taken down as zero When treating the data the mean values of the second to fifth ejacualtes have been used

Each animal was studied for about 2 months During this time seasonal variations in the seminal characteristics (Cf KIHLSTRÖM) occurred in 2 animals one injected at a maximum one at a minimum in the sexual cycle In the tables the p values obtained when data from these individuals have been included are given in brackets The data from the rabbit giving urine in most ejaculates have been excluded altogether from the tables

The statistical analysis has been performed according to a subsample test designated by SNEDECOR (1946)

Results

In the present material no difference in the response between animals receiving the different doses of the hormone could be detected As seen from Table I oxytocin significantly increases the volume of the first ejaculate in a series yielded immediately after an injection As the total amounts of sperm cells in these ejaculates are unchanged compared with the controls the increased volume must depend upon a larger amount of seminal fluid In keeping with this the density of sperm cells is lowered (Table I) However if data from the 2 animals showing seasonal variations are included there appears a larger amount of sperm cells in the first ejaculate (Table I)

The mean volumes of ejaculates 2—5 on the other hand are not influenced by oxytocin (Table II) nor is the total volume yielded during 30 min after a hormone injection significantly larger than after a control injection

Ejaculates 2—5 in a series taken after a hormone injection have larger amounts and higher densities of sperm cells than corresponding ejaculates taken after a saline injection (Table II) However the total amounts of spermatozoa yielded during 30 min after an injection are the same irrespective of

Table I. Seminal characteristics from first ejaculates yielded by 6 animals in exhaustion experiments performed on the day after maxima and minima in the sexual cycle

	Control inj. 11 after maxima 10 after minima	Hormone inj. 12 after maxima 8 after minima	p-value
Volume, ml	0.33 ± 0.031	0.47 ± 0.039	< 0.005 (< 0.005)
Sperm density, no. of cells $\times 10^7$/ml	175 ± 27.7	119 ± 19.4	≈ 0.05 (> 0.1)
Sperm amount, no. of cells $\times 10^7$	56 ± 8.4	54 ± 8.6	– (< 0.01)

Mean values ± SE.

In brackets p-values obtained when data from the 2 animals demonstrating seasonal variations have been included.

Table II. Seminal characteristics from the ejaculates 2—5 yielded by 6 animals in exhaustion experiments performed on the day after maxima and minima in the sexual cycle

	Control inj. 13 after maxima 10 after minima	Hormone inj. 12 after maxima 11 after minima	p-value
Volume, ml	0.11	0.11	–
Sperm density, no. of cells $\times 10^7$/ml	313 ± 38.4	431 ± 35.0	< 0.025 (< 0.005)
Sperm amount, no. of cells $\times 10^7$	48 ± 5.7	66 ± 5.0	< 0.025 (< 0.01)

Mean values ± SE.

In brackets p-values obtained when data from the 2 animals demonstrating seasonal variations have been included.

whether oxytocin or a saline solution has been injected. This observation suggests that the available store of spermatozoa is emptied more rapidly under the influence of the administrated hormone.

When the hormone injections were performed after a minimum in the sexual cycle sperm density in ejaculates 2—5 was 571 ± 52 ($\times 10^7$/ml) as compared with 304 ± 42.2 ($\times 10^7$/ml) when the injections were done after a maximum ($p < 0.001$). The corresponding values in sperm count were 64 ± 8.6 and 50 ± 5.6 ($\times 10^7$) ($p < 0.005$). There were no significant differences for the controls in these respects. The observations may indicate that the oxytocic effect varies with the androgenic status of the animal.

In addition to these rapidly occurring effects some facts demonstrate a more prolonged action of the hormone Thus the day after an injection sperm density was 70 ± 8.8 ($\times 10^7$/ml) in the oxytocin treated animals and 128 ± 21.9 ($\times 10$ /ml) in the controls ($p < 0.01$) The sperm count shows the same tendency ($p < 0.05$)

Besides the gelatinous masses of rabbit semen occur in 74 % of the cases the day after a hormone injection as compared with 43 % after control injection ($p < 0.05$)

Contamination with urine was frequently observed in connection with hormone injections Thus 11 first ejaculates out of 42 given after hormone injection contained urine (26 %) After the control injections only one ejaculate out of 41 contained urine (3 %)

Discussion

By the training performed the animals became familiar with the experimental procedures which did not seem to irritate them during the experiments

Apparently the immediate effect of oxytocin is an increased output of fluid from the accessory glands followed later on by a more rapid emptying of the vasa deferentia (and epididymis?) It may be supposed that as in females the smooth musculature of the internal genital organs is stimulated by oxytocin The observed effects of the hormone may then have the following explanation At the first ejaculation after the administration of oxytocin the muscles of the accessory glands became more intensively contracted thereby increasing the volume of liquid yielded In the following ejaculations the stimulating action of the hormone on vasa deferentia and possibly epididymis becomes noticeable in the increased amounts of sperms in the ejaculates immediately following upon the first one

The total amounts of sperms yielded during the whole exhaustion test is nearly the same in the hormonetreated animals and the controls This might mean that the available store of spermatozoa in the genital organs is efficiently emptied during the experiments whether or not the animals have been injected with oxytocin The effects of the hormone are thus an increased amount of seminal fluid and a changed distribution of the available spermatozoa among the ejaculates

Harris (1947) found that in the rabbit the amount of oxytocic principle liberated by the neurohypophysis on maximum stimulation was equivalent in oxytocic action to 0 2—0 5 I U of Pitocin Simon and Kardos (1954) estimated the amount of this principle in the pituitary of the rabbit to be 0 6—1 5 I U In comparison the doses used in the present investigation seem rather high However, in the rabbit the half life of oxytocin in the blood is low about 3 min (Chaudhury and Walker 1959) It is thus possible that the oxytocin concentration in the blood at the first ejaculation which took part about 45 sec after the injection was within the physiological range

There are some earlier reports on the influence of oxytocin upon male genital organs. Most authors have failed to demonstrate any obvious effects of oxytocin or extracts of the posterior lobe upon the vasa deferentia and/or the accessory glands (WADDELL 1916, PERUTZ and MERDLER 1924, MARTINS and VALLE 1939, CROSS 1955, 1959, CROSS and GLOVER 1958). Recently DZIUK and NORTON (1962), using exactly the same procedure as the present authors obtained no effects using the very high dose of 50 I.U. However, they gave subcutaneous injections supposing that the hormone was absorbed after 20 min when the semen collections were performed. Some investigators have found effects in certain species only (PERUTZ and MERDLER 1924) or in oestrogen treated animals (DE JONGH quoted from LAQUEUR, DE JONGH and TAUSK 1948). WADDELL (1917) has observed an increased motility of the uterus masculinus of the rabbit using Pituitrin. In a preliminary paper EWY and BIELANSKI (1962) have reported in the ram an increased rate of transport of spermatozoa through the ductus deferens after intravenous injection of oxytocin.

The works of SHIBUSAWA *et al.* (1955), ARMSTRONG and HANSEL (1958) and MARTINI *et al.* (1958) suggest that posterior pituitary polypeptides incite a release of gonadotrophins from the anterior lobe. In the present work the gelatinous component of the ejaculates occurred most frequently the day after a hormone injection. As the production of this substance is stimulated by androgens (CHENG and CASIDA 1949, CHENG *et al.* 1950, PARSONS 1950) this observation may indicate that the oxytocin via the gonadotrophins increases the concentration of androgens in the blood. The low sperm density and sperm count found on the day following upon a hormone injection may also be ascribed to increased amounts of androgens retarding the functions of epididymis and vasa deferentia (MARTINS and VALLE 1939).

The lower densities and amounts of sperm cells found when the injections were performed after a maximum in the sexual cycle indicate that the effect of oxytocin is influenced by other sex hormones. However, it is possible that the high doses used partly mask such synergistic actions.

Studies of the effects of the hormone on individual organs in situ and in vitro are in progress.

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Acta physiol scand 1963 59 370—377

From the Institute of Zoophysiology University of Uppsala Sweden

Cyclic Phenomena in the Sexual Functions of the Bull

By

J E Kihlström

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Abstract

Kihlström J E *Cyclic phenomena in the sexual functions of the bull* Acta physiol scand 1963 59 370—377 — By means of statistical methods cyclic variations in some sexual functions of ten bulls used for artificial inseminations have been demonstrated The cycles found have a length of a few weeks and indicate cyclic processes in the epididymis as well as in the accessory glands The cyclic variations in fertility were more regular in young bulls than in older ones

The existence in both sexes of a similar arrangement of interacting gonadotrophic and gonadal hormones makes it plausible to expect the occurrence of cyclic phenomena corresponding to the oestrous cycle also in males Nevertheless such phenomena have apparently not been observed until the last decade (Doggett 1956 Kihlström 1958 Degerman and Kihlström 1961, Kihlström and Degerman 1962) However the observations reported all refer to rabbits for which cyclic variations have been established in some characteristics of the semen (Doggett 1956 Kihlström 1958 Degerman and Kihlström 1961 Kihlström and Degerman 1962) and in the libido (Degerman and Kihlström 1961) The present paper describes a first attempt to reveal sexual cycles if existing in bulls by utilizing the enormous amount of data concerning fertility and semen characteristics accumulated by the AI (artificial insemination) associations It must be kept in mind that bulls used for artificial insemination are rigidly selected in order to fulfill great demands upon sexual functions

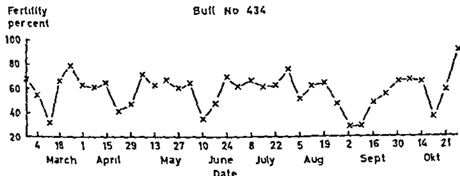


Fig 1 Weekly variations in the fertility of the ejaculates of one bull

Material and Methods

Fertility and semen characteristics were studied in 32 bulls of the Swedish Red Breed belonging to the A I Association at Eskilstuna The quantity of data necessary for the statistical treatment applied requires that the animals have been used for collection of semen regularly without interruptions for about 7 months Ten bulls five of them during two different periods fulfilled these conditions and could consequently be utilised for the present investigation

Semen was collected according to ordinary veterinary practice With a few exceptions the bulls were put to service regularly every seventh day and generally two or in a few cases three ejaculates were obtained from the same bull within approximately 0 min The quality of the ejaculates was assessed by the veterinary staff by measuring the volumes and by observing density mass activity and motility By multiplying the volumes of the ejaculates by their respective densities of sperm cells approximate expressions of the number of spermato oa per ejaculate were obtained

If considered of good quality the ejaculates yielded by the same bull on one occasion were pooled before being used for insemination Except fertility all characteristics of semen studied refer to the first ejaculate given in such a s ries

Fertility was defined as the number of observed pregnancies in per cent of the number of first inseminations performed Only ejaculates used for more than 20 inseminations have been included As discussed earlier (KIHLSTROM 1958) this number of inseminations will give a reliable index of the fertility of the semen samples The volume of the semen yielded was measured in graduated test tubes with an accuracy of 0 2 ml However some semen always remains in the artificial vagina thus increasing the error in the figures obtained The mean volume of all ejaculates is 5 3 ml Density and mass activity were subjectively estimated according to a three graded scale motility according to a four graded scale

The length of the cycles was determined by arranging the collected data in chronological order with a constant time interval Part of a series thus obtained is diagrammatically illustrated in Fig 1 The series was then rearranged in horizontal rows of identical length one below the other This arrangement was repeated the number of figures in each row varying from 3 to 10 (Cf WORTHING and GEFFNER 1944 p 296) In all series every minimum value irrespective of its deviations from the adjacent values was marked When the minimum was broad and comprised two or more identical values the most central value was looked upon as the real minimum If the minimum consisted

Table I Cyclic variations in fertility

Bull no	Age in years at the end of the studied period	Per cent of rejected ejaculates	Length in weeks of significant cyclic variations	Most pronounced cyclic variation			
				Length in weeks	t	f	p<
451	3 0	0	3 6	3	1 99	54	0 05
451	7.3	2	4 6 8 10	4	2 05	73	0 025
445	3 8	11	5	5	2 49	51	0 01
446	3 8	15	—	7	1 29	30	0 2
450	3 8	0	—	3	1 54	35	0 1
450	6 3	11	3 6 9	6	2.22	63	0 025
444	4 8	0	4 5 8	8	2 33	38	0 025
448	5 0	0	6	6	2 18	33	0 025
434	7 0	3	—	6	1 36	35	0 1
434	9 0	2	7	7	11 48	57	0 0005
431	7 8	2	8	8	1 84	55	0 05
431	12 0	0	—	6	1 33	30	0 1
426	8 8	0	8 9	8	2 34	46	0 025
426	11 8	6	4	4	1 71	48	0 05
418	10 3	4	9	9	1 74	55	0 05

of an even number of figures the two most central values were marked as the minimum At the calculations each figure in such a couple was looked upon as half a minimum In a preliminary paper (KIHLSTROM 1962) the maximum values were used instead of the minimum values However the minimum values have proved to be more regular

Unless the collected data express cyclic variations the minimum values will be distributed at random among the vertical columns obtained by the procedure described However a cyclic variation of a given length appears as an accumulation of the majority of the minima in one and the same column This happens when the horizontal row has a length approaching that of the cycle or a multiple of the cycle The longest period investigated in this way amounts to 10 times the interval between the figures which is one week.

The probability of a given frequency of minima in a vertical column to be statistically different from that expected if the minimum values in the whole series were distributed at random among the columns, has been tested according to the known formula

$t = (p_m - p) / \sqrt{p_m q_m / n_m + p q / n}$

p_m = the relative frequency of minima in the vertical column exhibiting the biggest number of minima when the horizontal row contains m consecutive data

$q_m = 1 - p_m$

n_m = the number of data in the column

p = the relative frequency of minima in the whole series

$p = 1 - p_m$

n = the number of data in the whole series

An approximate expression of the regularity in the occurrence of the minima was arrived at by the following procedure When the length of the rows described above

Table II Cyclic variations in volume and amount of sperm cells per ejaculate

Bull no	Age in years at the end of the studied period	Volume					Amount of sperm cells per ejaculate				
		Length in weeks of significant cyclic variations	Most pronounced cyclic variations				Length in weeks of significant cyclic variations	Most pronounced cyclic variations			
			Length in weeks	t	r	p<		Length in weeks	t	r	p<
451	3 0	3 6 10	10	2 63	44	0 01	3 6 8 9	9	2 59	43	0 01
451	7 3	4 8 10	8	2 59	65	0 01	4 8 10	8	2 59	65	0 01
445	3 8	5 10	10	2 98	46	0 005	5 10	10	2 98	46	0 005
446	3.8	—	7	1 58	29	0 1	7	7	2 67	29	0 01
450	3 8	—	7	1 48	38	0 1	4 5 6	6	2.59	37	0 01
450	6 3	6 9 10	10	2 72	59	0 005	7 10	10	1 89	60	0 05
444	4 8	—	3	1 45	33	0 1	6	6	1 86	36	0 05
448	5 0	4 5	5	1 86	33	0 05	5	5	3 22	33	0 005
434	7 0	—	6	0 78	37	0 3	—	5	1.22	34	0 2
434	9 0	—	6	1 65	54	0 1	—	6	2 19	54	0 025
431	7 8	4 6 8 9	8	2 15	52	0 025	4 6 8 9	4	2.51	55	0 01
431	12 0	5	5	2 00	32	0 05	—	4	1 21	33	0.2
426	8 8	6	6	1 76	48	0 05	8	8	1 83	46	0 05
426	11 8	—	4	2 01	62	0 075	—	5	1 04	41	0.2
418	10 3	4 6 8 10	10	3 13	55	0 005	6 8 9 10	8	2 19	56	0 025

approximated the length of the cycle i e the row gives the most pronounced cycle the highest number of minima accumulated in one column was divided by the number of minima in the whole series The figure thus obtained represented the relative frequency of regularly occurring minima

In order to study whether or not the different cyclic variations found coincide chronologically a chi square test was applied The observed numbers of minima of fertility occurring simultaneously with minima of the remaining characteristics have been compared with the numbers expected if the minima were distributed at random The same procedure was applied also to the occurrence of the maxima

Results

All bulls studied four of them during two periods show significant rhythmicities in at least one of the characteristics studied (See Table I and II)

Fertility Nine out of ten bulls two of them during two different periods show significant cyclic variations in the fertility of the pooled ejaculates (Table I) There is a relationship between the regularity of these cyclic variations and the age of the animals Fig 2 is obtained by plotting the relative frequency of regularly occurring minima against the age of the animal The diagram demon

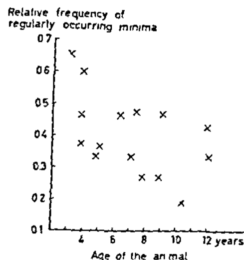


Fig 2 Relative frequency of regular occurring minima in fertility plotted against the age of the animal

strates that regularity of the cycle is most pronounced in young animals and decreases with increasing age according to a linear relationship ($r = -0.52$ $t = 2.19$ $p < 0.025$)

It must be pointed out that only semen deemed to be of good quality has been used for insemination Most likely this selection of samples diminishes the available informations about the cyclic variations The percentage of rejected ejacualtes in each bull is given in Table I

Volume Seven of the ten bulls show significant periodic variations in the volumes of the first ejaculate in a daily series (Table II) In these cases no relationship between the characteristics of the cycles and the age of the animals could be detected

As discussed above the accuracy in the determinations of volume and fertility are comparatively good However density of sperm cells mass activity and motility are only roughly estimated according to a three or four graded scale For this reason possibly existing cyclic variations in these characteristics must be very pronounced in order to be detected Nevertheless all bulls studied show significant periodical variations in the number of sperm cells per ejaculate at least during one studied period in each bull Table II) Sporadically there are weakly significant variations also in density and motility of the sperm cells

As seen from Table III maxima as well as minima in the different characteristics studied coincide significantly more often than what should be expected in a random distribution Consequently the cycles found in fertility volumes of the ejaculates density amount and motility of spermatozoa all are to a certain degree synchronous

Table III Comparison between observed and expected frequencies of simultaneously occurring phases of cyclic variations in different characteristics

Simultaneous phases		Observed frequency	Expected frequency	χ^2		p<
Volume	*Fertility*					
Maximum	Maximum	68	51 9	4 69	6 80	0 01
	Not maximum	99	115 1	2 11		
Minimum	Minimum	73	53 6	6 66	9 63	0 005
	Not minimum	101	120 4	2 97		
Amount of sperm cells	*Fertility*					
Maximum	Maximum	66	54 7	2 13	3 09	0 1
	Not maximum	110	121 3	0 96		
Minimum	Minimum	73	53 3	6 92	10 00	0 005
	Not minimum	100	119 7	3 08		
Density of sperm cells	*Fertility*					
Maximum	Maximum	30	19 6	4 23	6 14	0 025
	Not maximum	33	43 4	1 91		
Minimum	Minimum	37	25 9	4 34	6.27	0 025
	Not minimum	47	58 1	1 93		
Motility	*Fertility*					
Maximum	Maximum	48	37 3	2 79	4 05	0 05
	Not maximum	72	82 7	1.26		
Minimum	Minimum	41	36 6	0 42	0 60	—
	Not minimum	78	82 4	0 18		

Discussion

Unfortunately the long interval between the ejaculations in the present material does not permit a reliable estimate of the lengths of the cycles Fig 3 shows the distribution of the lengths of all cycles determined as the interval in weeks between two successive minima The mean length of these cycles is 2 9 weeks The mean length of the significant cyclic variations on the other hand is 6 4 weeks (Table I—II) suggesting that the found lengths of these variations might be multiples of the length of the primary cycle However some bulls show significant cyclic variations with a length of 3 or 4 weeks Therefore it is also possible that a cycle having this length is superimposed by a longer one By way of comparison may be mentioned that the average length of the oestrous cycle of the cow is 21 days (ECKSTEIN and ZUCKERMAN 1956) From time to time random variations also cause minimum values interfering with those of

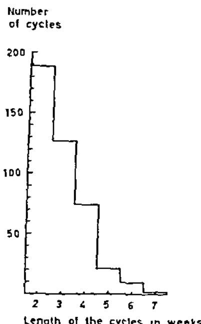


Fig. 3. The observed distribution of the lengths of cycles.

the male sexual cycle. Therefore a third possible explanation is that at least in some bulls the endogenous cycle has a length of 5 to 6 weeks, the shorter intervals between minima being caused by random variations. In order to get further information about the length of the cycle and the influence of age a bigger and more suitable material including bulls used for service at shorter intervals is being studied.

As *significant cyclic variations are found in the amounts of sperm cells* as well as in the volumes of the ejaculates, it must be concluded that cyclically varying processes occur in the epididymis as well as in the accessory sex glands.

Veterinary experience clearly shows that semen characteristics may vary considerably depending upon the skill of the person colllecting the semen. In the present material two veterinarians have performed the collections, one of them 4 or 5 days a week, the other 1 or 2 days a week. However, there are no significant differences in the semen characteristics between ejaculates collected by these two persons. Consequently *the cycles found are not caused by differences in skill between these persons*.

Work is in progress with a view of elucidating the physiological background of the cycles found.

I thank the A. I. Association of Eskilstuna and especially its chief veterinarian Dr I. Strö[illegible] for placing records at my disposal. My thanks are due to Professor P. E. Lindahl for reading the manuscript and thereby giving valuable suggestions. I thank Mrs Elisabeth V[illegible] and Eva Carlsson for technical assistance. Financial support from the Lars Hierta Memorial Foundation is gratefully acknowledged.

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Acta physiol scand 1963 59 378—389

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Enzymatic and Molecular Properties of Cholinesterases in Human Liver

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Abstract

SVENSMARK O *Enzymatic and molecular properties of cholinesterases in human liver* Acta physiol scand 1963 *59* 378—389 — Three fractions of human liver acylcholine acyl hydrolase (cholinesterase EC 3 1 1 8) were separated by chromatography on DEAE cellulose and by gel filtration on Sephadex G 200 One fraction (II) was identical with human serum cholinesterase with respect to enzymatic properties (substrate and inhibitor specificity pH dependence activation by ammonium ions) to solubility in the presence of zinc ions and to electrophoretic mobility before and after removal of sialic acid Since as much blood as possible was removed from the liver before extraction it seems unlikely that the fraction originates from plasma The other fractions (Id and Ie) were also identical with serum cholinesterase with respect to enzymatic properties but differed with respect to molecular properties They were not sialoproteins and their electrophoretic mobilities differed from that of native and of neuraminidase treated serum cholinesterase Separation on Sephadex G 200 indicates a lower molecular weight of fraction Ie than of fraction Id and II The findings are consistent with the assumption that serum cholinesterase is produced in the liver with fraction Id and Ie as precursors and fraction II as the final product

Cholinesterase activity was demonstrated in the liver of the cat dog and rabbit by PLATTNER and HINTNER (1930) The activity is mainly due to acyl choline acyl hydrolase (*cholinesterase* EC 3 1 1 8)[1] *i e* an enzyme of the same type as serum cholinesterase (SAWYER 1945 SAWYER and EVERETT 1947 AUGUSTINSSON 1948) It is generally assumed that serum cholinesterase is

[1] Report of the Commission on Enzymes of the International Union of Biochemistry I U B Symp Series no 20 Pergamon Press Oxford 1961

produced in the liver (FABER 1941 SAWYER and EVERETT 1947, GEREBTZOFF 1959) This view is based on the finding of a decrease in the activity of serum cholinesterase in liver disease (ANTOPOL, SCHIFRIN and TUCHMAN 1937) and after experimental liver damage by carbon tetrachloride (BRAUER and ROOT 1946) Knowledge of the properties of human liver cholinesterase is scarce β carbonaphthoxycholine is split by the enzyme and in starch gel electrophoresis at pH 7 2 it migrates in a broad zone comprising components of identical and lower mobilities than the mobility of the serum enzyme (ECOBICHON and KALOW 1962)

In the experiments presented in this report the properties of human liver and serum cholinesterases were compared Their enzymatic properties were investigated particularly with respect to substrate and inhibitor specificity, and their molecular properties with respect to electrophoretic mobility before and after treatment with neuraminidase

Materials and Methods

Eight human livers were obtained 5—24 hours after death (accidents coronary occlusion) The liver cholinesterases were prepared from aqueous extracts by chromatography on diethylaminoethyl cellulose (DEAE cellulose) gel filtration on Sephadex G 200 and by preparatory electrophoresis

Extraction The liver was cut into pieces of about 0 5 cm^3 and larger vessels were excised To remove as much blood as possible the tissue was rinsed three times in 0 9 per cent sodium chloride and each time squeezed gently between two layers of filter paper The tissue was then homogenized in a WARING blendor with two volumes of water The homogenate was centrifuged at 14 000 × g for 30 min and the precipitate discarded The supernatant was adjusted to pH 5 2 with 1 M acetic acid and centrifuged at 14 000 × g for 30 min The precipitate was discarded and the supernatant adjusted to pH 7 0 with 1 M sodium hydroxide and dialyzed against running tap water for 24 hours A slight precipitate formed during dialysis was removed by centrifugation These operations were performed at 5—8 C The resulting supernatant was clear or slightly turbid The Copenhagen tap water contains relatively large amounts of calcium (2 5 mM) and precipitation of calcium phosphate on the DEAE cellulose might have influenced the chromatographic separation However chromatograms obtained with liver extracts dialyzed against distilled water could not be distinguished from those obtained with the calcium containing extracts

Chromatography on DEAE cellulose (WHATMAN DE 50) was carried out at room temperature (20—22 C) on 80 cm × 5 cm^2 columns which were eluted with linear salt gradients from 25 mM NaH_2PO_4 pH 7 to 25 mM NaH_2PO_4 pH 7 + 0 3 M NaCl The eluates were collected in 25 ml fractions and their conductivity determined (Conductivity meter CDM 2 Radiometer Copenhagen) The different cholinesterase fractions were concentrated by precipitation with 3 M ammonium sulphate The precipitate was collected by centrifugation and dissolved in the smallest possible volume of distilled water

Gel filtration The dissolved precipitate from DEAE chromatography was applied on a 30 cm × 3 2 cm^2 column of Sephadex G 200 (Pharmacia Uppsala Sweden) and eluted with 0 1 M NaCl at 12 ml/h at room temperature The eluate was collected in 6 ml fractions The cholinesterase fractions were concentrated by precipitation with 3 M am

monium sulphate The precipitate was dissolved in the smallest possible volume of distilled water and desalted on a column of Sephadex G 25

Preparatory electrophoresis was carried out on a column (100 cm × 5 cm²) packed with ethanolyzed cellulose (Munktell Sweden) in Tris buffer (50 mM tris (hydroxymethyl) aminomethane 50 mM NaCl adjusted to pH 8 6 with 6 M HCl) An electric field of 3 V/cm was applied for 70 hours at 10 C and the eluate was collected in 5 ml fractions Before application on the column the protein solution was transferred to the Tris buffer on a column of Sephadex G 25

The *protein concentration* was estimated by continuous recording of the light transmission at 254 mμ (Uvicord light path 3 mm LKB Stockholm) or by measurement of the optical density at 280 mμ in 0 1 M phosphate buffer pH 7 3 in silica cells of 10 mm light path (BECKMAN spectrophotometer DU or DB)

The *cholinesterase activity* was determined *colorimetrically* in (a) chromatographic eluates of low enzyme activity and (b) in cholinesterase preparations when different substrates were used

(a) *Assay of eluates* 50 μl of eluate were incubated at 30 C for 1 hour with 1 ml of 2 5 mM butyrylcholine iodide in 0 25 M sodium phosphate buffer at pH 7 3 After the incubation period the concentration of butyrylcholine was determined by the procedure of HESTRIN (1949)

(b) Enzyme activity with *different substrates* was determined by incubation at 30 C with 4 ml of 12 5 mM acetylcholine iodide butyrylcholine iodide or benzoylcholine chloride in 0 25 M sodium phosphate buffer at pH 7 3 At 15 min intervals 1 ml samples were withdrawn and the concentration of the choline ester determined colorimetrically Both in the assay of eluates and in the determination of the enzyme activity with different substrates correction for spontaneous hydrolysis was obtained from a sample prepared with water in lieu of enzyme

The *cholinesterase activity* was determined *spectrophotometrically* in (c) the assay of eluates of sufficiently high activity and (d) in the determination of inhibitor specificity (e) pH dependence and (f) ammonium chloride activation of liver cholinesterase preparations A BECKMAN spectrophotometer DB with a BECKMAN logarithmic potentiometer recorder was used with silica cells of 1 cm light path The cell compartment was maintained at 30 ± 0 3 C by a water thermostat The final concentration of the substrate benzoylcholine chloride was 0 1 mM The optical density at 240 mμ was recorded and the cholinesterase activity calculated in μmoles/min/ml as

$$\Delta E_{240}/\text{min} \times 0\ 16\ v/c$$

where v is the volume of the reaction mixture and c the volume of the sample The factor 0 16 was determined from $0\ 1/\Delta E_{240}$ in experiments where all substrate (0 1 mM) was split (10 expm S D = 0 006)

(c) In *assay of eluates* the cell contained 1 ml substrate in 0 08 M sodium phosphate buffer at pH 7 3 and 10 μl of the eluate

(d) When *inhibitor specificity* was studied the cell contained 0 8 ml of substrate in 0 08 M sodium phosphate buffer at pH 7 3 0 1 ml water or inhibitor and 0 1 ml enzyme The following inhibitors were used in aqueous solutions Neostigmine (Prostigmine bromide© Hoffmann La Roche) Astra 1397 (10-(α-diethylaminopropionyl) phenothiazine chloride Astra Sodertalje Sweden) and succinylcholine chloride (Fluka) Neostigmine was incubated with the enzyme for 30 min before the substrate was added

(e) The pH activity curve was investigated with 67 mM Na_3PO_4 adjusted to different pH values between 5 5 and 9 0 with HCl Correction for spontaneous hydrolysis was obtained with water in lieu of enzyme

(f) The *activation* by ammonium chloride was studied in 5 mM *sodium phosphate* buffers containing 0—0 8 M NH_4Cl *adjusted to pH 7 3*

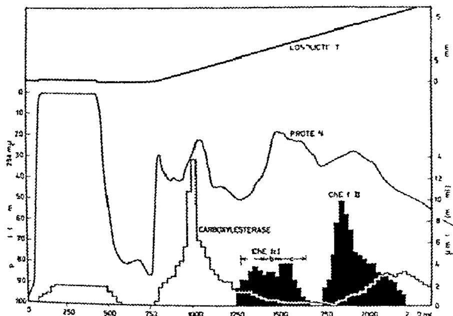


Fig 1 *DEAE cellulose chromatography of liver extract* (500 ml) The curve marked protein gives the protein concentration in the eluate determined by recording of the transmission at 254 mμ Black columns Cholinesterase (ChE) activity in μ moles butyrylcholine/ml min at pH 7 3 and 30 C. White columns Carboxylesterase activity in μmoles phenyl acetate/ml min at pH 7 3 and 30 C. Upper curve Conductivity of eluate Abscissa ml eluate

All liver cholinesterase fractions were contaminated by carboxylic acid hydrolase (*carboxylesterase* EC 3 1 1 1) The activity of carboxylesterase was determined spectrophotometrically by the procedure used by ZELLER (1956) for the determination of cholinesterase activity Phenyl acetate and phenyl butyrate were used as substrates The equipment was the same as used in the spectrophotometric determination of cholinesterase described above The cell contained 1 0 ml of 2 mM phenyl acetate or phenyl butyrate in 25 mM Tris buffer at pH 7 3 10 μl of the enzyme and 10 μl of 0 1 M Astra 1397 to inhibit the cholinesterase The optical density was recorded at 280 mμ and the enzyme activity calculated in μmoles/min/ml as

$$\Delta E_{280} /\text{min} \times 1\ 64\ v/e$$

where v is the total volume (1 02 ml) and e the volume of enzyme (10 μl) The factor 1 64 was determined from $1/\Delta E_{280}$ in experiments where 1 mM substrate was split (10 expm S D = 0 02)

The *electrophoretic mobility* of liver cholinesterase was determined in paper electrophoresis with corrections for evaporation electro-osmosis paper structure and adsorption of protein to the paper (WALDMANN MEYER and SCHILLING 1959 SVENSMARK and KRISTENSEN 1963) Cholinesterase was stained with β carbonaphthoxy choline (Sigma) (RAVIN TSOU and SELIGMAN 1951) as the α naphthyl acetate previously used (SVENSMARK 1961) stains carboxylesterases

Treatment of liver cholinesterases with N acetylneuraminate glycohydrolase (*neuraminidase* EC 3 2 1 18) (Neuraminidase Behringwerke Marburg Germany) was carried out at 5 C as described previously (SVENSMARK and KRISTENSEN 1963)

Table I. Recovery of butyrylcholine splitting cholinesterases in the extraction procedure and separation of fraction I and II in chromatography on DEAE cellulose. The values represent the mean of duplicate determinations. For livers no. 1 and 2 complete quantitative data are not available.

Liver no.	Cholinesterase activity (μmoles butyrylcholine/g tissue wet weight/h)			
	Homogenate	Extract	Chromatographic fractions	
			I	*II*
3	60	28	8	12
[1]3	60	22	9	8
4	[illegible]8	35	12	13
4	58	40	13	13
5	63	23	—	—
6	1[illegible]2	69	14	9
6	118	43	14	14
6	—	43	8	12
6	117	49	—	—
7	86	38	13	12
8	—	40	8	11
8	—	40	7	11

[1] After storage of the extract at −5 C for 4 months.
[2] After storage of the homogenate at 25 C for 7 days.

To compare the properties of liver cholinesterases with those of human serum cholinesterase a preparation was made by fractional precipitation with ammonium sulphate and DEAE cellulose chromatography. The purification factor was 35 (SVENSMARK and KRISTENSEN 1963).

Results

Separation of different liver cholinesterases

By DEAE cellulose chromatography of aqueous extracts of liver two main fractions of cholinesterase (fraction I and II) were separated. Fig. 1 shows a typical experiment; similar results were obtained in 16 experiments with extracts from 8 livers. The activities of the two fractions were about equal (Table I). Storage of the extracts for several months at —5 C or of the homogenate at 25 C for 7 days did not alter the total or fractional activities (Table I).

Fraction I was inhomogeneous with three partially separated peaks of cholinesterase (Fig. 1, fraction Ia, Ib, Ic). A partial or complete separation of fraction I was also obtained in gel filtration on Sephadex G 200 (Fig. 2). One fraction (d) appeared immediately after the void volume and another (e) was retarded on the column indicating a higher molecular weight of fraction Id than of fraction Ie. Gel filtration of the chromatographic fraction II of liver

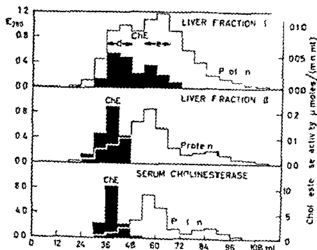


Fig 2 Gel filtration on Sephadex G 200 of the chromatographic fraction I fraction II and of human serum cholinesterase White columns Protein concentration of the eluate (E) Black columns Cholinesterase (ChE) activity in μmoles benzoylcholine/ml/min at pH 7 3 and 30 C Abscissa ml eluate

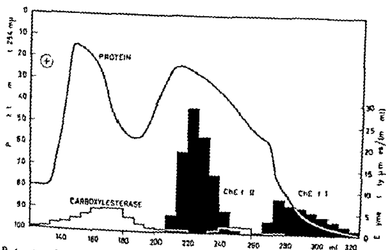


Fig 3 Preparatory electrophoresis at pH 8 6 of a chromatographic fraction I contaminated with fraction II The curve marked protein gives the protein concentration (per cent transmission at 254 mμ) Black columns Cholinesterase (ChE activity in μmoles butyrylcholine/ml/min at pH 7 3 and 30 C White columns Carboxylesterase in μmoles phenyl acetate/ml/min at pH 7 3 and 30 C Abscissa ml eluate

cholinesterase and of human serum cholinesterase indicates that their molecular size is of the same order as fraction Id (Fig 2)

In preparatory electrophoresis at pH 8 6 the liver cholinesterases were separated into the same two fractions as in chromatography on DEAE cellulose The fraction corresponding to the chromatographic fraction II migrated faster towards the anode than fraction I There was no evidence of in geneity of fraction I (Fig 3)

Table II Recovery of cholinesterase activity in extraction and separation procedures

	No of expm.	Recovery per cent
Extraction	10	35–65
Chromatography on DEAE cellulose	10	30–75
Gel filtration on Sephadex G 200	5	45–60
Electrophoresis of fraction I	3	15–25
Electrophoresis of fraction II	3	75–80

Table III Specific activities of cholinesterases and carboxyl sterase in different fractions from human liver (µmoles/min ml E_{280}) Cholinesterase was determined with benzoylcholine as substrate carboxylesterase with phenyl acetate The values represent the mean of duplicate determinations on preparations obtained in a typical experiment

Fraction		Cholinesterase	Carboxylesterase
Liver extract		0 009	0 34
Chromatographic fractions	Ia	0 007	0 50
	Ib	0 008	0.20
	Ic	0 004	0 07
	II	0 012	0.27
Fractions obtained in preparatory electrophoresis	*I*	0 060	0 47
	II	*0 029*	*0 09*
Fractions obtained in gel filtration	Id	0 031	0 97
	Ie	0 074	0 4
Supernatant after precipitation of the chromatographic fraction II with 10 mM zinc acetate at pH 7		0 300	0.20

The recovery of enzymatic activity in the extraction and the different separation procedures are given in Table II

In chromatography on DEAE cellulose three or four fractions of non-choline esterases appeared They were not activated by 10 mM $CaCl_2$ were not inhibited by 10 mM ethylenediamine tetraacetic acid and they hydrolyzed phenyl butyrate 2—5 times faster than phenyl acetate This indicates that the non-choline esterase fractions were carboxylesterases (formerly ali-esterase or B-esterase for references see HOFSTEE 1960) Complete separation of cholinesterase and carboxylesterase was not attempted their specific activities in the different fractions are given in Table III

Cholinesterase fraction I was precipitated by 10 mM zinc acetate at pH 7 whereas fraction II remained in solution as did serum cholinesterase (COHN 1953) More than 95 per cent of the carboxylesterase was precipitated by zinc ions

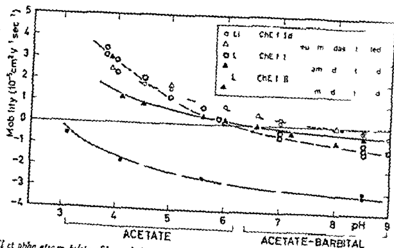


Fig 4 *Electrophoretic mobility of liver cholinesterases as a function of pH* (1 C) The buffer composition is indicated on the abscissa the ionic strength varied in each experiment between 0 08 and 0 1 Liver cholinesterase (ChE) fraction II was obtained in chromatography on DEAE cellulose fraction Id and Ie in gel filtration The mobilities of native and neuraminidase treated liver fraction II were identical with those of native and neuraminidase treated human serum cholinesterase (lower and upper full line respectively SVENSMARK and KRISTENSEN (1963) Each dot represents a single determination

Properties of liver cholinesterases

The *electrophoretic mobility* at pH 4 to 9 was determined before and after treatment with neuraminidase for fractions Id and Ie obtained in gel filtration and for fraction II obtained in chromatography (Fig 4) The mobility pH dependence of fraction II was identical with that of human serum cholinesterase After treatment with neuraminidase the mobility of fraction II was changed in the same way as that of serum cholinesterase The mobility of the high molecular weight fraction Id was about parallel with that of neuraminidase treated fraction II (or serum cholinesterase) and the isoelectric point was at pH 8 The isoelectric point of the low molecular weight fraction Ie was at pH 6 and the change in mobility per unit of pH exceeded that of neuraminidase treated serum cholinesterase consistent with a lower molecular weight of fraction Ie than of the serum enzyme or of liver cholinesterase fraction II

Of the chromatographic fractions Ia and Ib showed the same mobilities as the high molecular weight fraction Id and fraction Ic the same mobility as the low molecular weight fraction Ie Treatment with neuraminidase did not change the mobilities of the chromatographic fractions Ia Ib and Ic

The *enzymatic properties* of the chromatographic fraction II of fractions Id and Ie obtained in gel electrophoresis and of fraction I and II obtained in preparatory electrophoresis were the same as those of human serum cholinesterase The relative activities of liver cholinesterases and of the serum enzyme were the same with 10 mM acetylcholine butyrylcholine and benzoylcholine as substrates

Table IV Substrate specificity of human serum and liver cholinesterase Fraction I and II were obtained by preparatory electrophoresis Substrate concentration 10 mM pH 7 3 30 C Relative activity is enzyme activity expressed in units of the activity determined with acetylcholine as substrate The values represent the mean of duplicate determinations

Preparation	Butyrylcholine		Acetylcholine		Benzoylcholine	
	µmoles/ml/h	Relative activity	µmoles/ml/h	Relative activity	µmoles/ml/h	Relative activity
Human serum cholinesterase	19 7	2 56	7 7	1	4 2	0 55
Human liver cholinesterase						
Fraction I	35 7	2 57	14.2	1	7 9	0 56
Fraction II	33 0	2 60	12 6	1	6 9	0 55

Table V Activity of liver and serum cholinesterase as a function of pH The liver fractions Id and Ie were obtained by gel filtration and fraction II by chromatography on DEAE cellulose Cholinesterase activity was determined with benzoylcholine as substrate The relative activity is expressed in units of the activity at pH 9 0 The values represent the mean of duplicate determinations

pH	Relative activity of cholinesterase			
	Serum (ChE)	Liver cholinesterase		
		Fraction Id	Fraction Ie	Fraction II
5 5	0 07	—	-	0 08
6 0	0 16	0 15	—	0 12
6 5	0.28	0 31	0 32	0.24
7 0	0 50	0 56	0 58	0 48
7.5	0 68	0 72	0 70	0 58
8 0	0 79	0.83	0 81	0 74
8.5	0 89	0 91	0 93	0.89
9 0	1 00	1 00	1 00	1 00

(Table IV) Changes in pH (Table V) inhibition with neostigmine Astra 1397 and succinylcholine (Fig 5) as well as activation with ammonium ions (Fig 6) had the same effect on the liver and the serum cholinesterases

Assuming a total recovery from the homogenate the cholinesterase activity of fraction II in liver extracts corresponds to a contamination with blood plasma of 5—15 per cent The dog liver — handled carefully so that the blood is not squeezed out — may contain as much as 13 per cent plasma (GIBSON *et al* 1946) In this study as much blood as possible was removed before ex

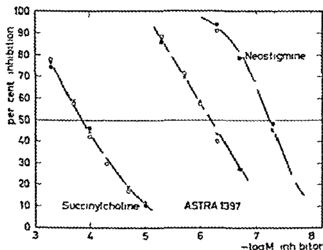


Fig. 5 *Inhibition of liver and serum cholinesterases as a function of the negative logarithm of inhibitor concentration* The liver fractions I and II were obtained in preparatory electrophoresis Cholinesterase activity was determined with benzoylcholine as substrate Fraction I (○) fraction II (●) and serum cholinesterase (×)

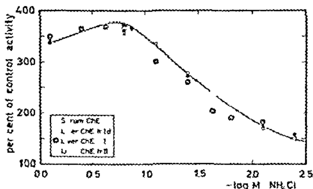


Fig. 6 *Activation of liver and serum cholinesterases by ammonium ions* The liver fraction II was obtained in DEAE cellulose chromatography and fractions Id and Ie in gel filtration The cholinesterase was determined with benzoylcholine as substrate

fraction and it seems unlikely that the cholinesterase of fraction II should originate solely from plasma

The cholinesterase activity of the different fractions I cannot originate from plasma The electrophoretic properties of these enzyme fractions were different from those of native and sialic acid free serum cholinesterase Furthermore incubation of serum cholinesterase preparations at 25 C and pH 5 5 for 10 days with equal volumes of liver homogenates did not lead to a decrease in mobility of the serum enzyme

Discussion

Before extraction of the liver as much of the blood as possible was removed to reduce the error from contamination with plasma It is quite possible that this procedure may have removed fractions present in the native tissue At any

rate two main fractions (I and II) of cholinesterases remained and were consistently found in the aqueous extract. Fraction II was identical with serum cholinesterase with respect to enzymatic properties electrophoretic mobility before and after removal of sialic acid and solubility in the presence of zinc ions It seems unlikely that this fraction originates solely from a contamination with plasma

Fraction I could be subdivided into two fractions (Id and Ie) in gel filtration on Sephadex G 200 Fraction Ie was retarded on the column indicating a lower molecular weight than that of fraction Id and II which appeared immediately after the void volume This is in accordance with the steeper slope of the mobility pH curve of fraction Ie than of fraction Id The electrophoretic mobilities of fraction Id and Ie were different from those of native and neuraminidase treated serum cholinesterase In contrast to serum cholinesterase (SVENSMARK 1961) they are not sialo-proteins since their mobilities remained unchanged by treatment with neuraminidase Fraction I and its subfractions did not exhibit the stability characteristic of sialo-proteins They were inactivated during preparatory electrophoresis and by standing at room temperature in the same way as sialic acid free human and equine serum cholinesterase (SVENSMARK unpublished observations)

The enzymatic properties of fraction I were identical with those of fraction II and serum cholinesterase This indicates that the three liver enzymes represent different molecular forms of the same enzyme and that the liver enzyme is identical with serum cholinesterase This finding is consistent with the assumption that the enzyme is synthesized in the liver fraction I representing precursors and fraction II the final product Standing of the liver homogenate at 25 C for one week did not lead to an increase of fraction I at the expense of fraction II indicating that fraction I is not due to degradation

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Cardiac Output in Healthy Subjects Determined with a CO Rebreathing Method

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Abstract

JERNERUS R G LUNDIN and D THOMSON *Cardiac output in healthy subjects determined with a CO_2 rebreathing method* Acta physiol scand 1963 *59* 390—399 — The cardiac output is measured with a CO_2 rebreathing method suggested by DEFARES (1956) The mixed venous CO_2 pressure is determined graphically from the breath by breath determined CO_2 content in a rubber bag during rebreathing from the bag Arterial CO_2 pressure is assumed to be the same as in end tidal air CO_2 eliminated per time unit is measured by collecting the expired air in a Douglas bag and the cardiac output is determined with the Fick equation For the CO_2 analysis a rapid infrared CO_2 meter is used The obtained values at rest are of the same size as those obtained with the Grollman acetylene method The values from the work experiments are of the same magnitude as those obtained with dye-dilution or direct Fick methods Stroke volume shows increase from rest to work about 60—80 per cent

The technique of using the lungs as an aerotonometer to measure the tension of gases in the mixed venous blood and hence the gas content of the latter was introduced by LOEWY and SCHROTTER as early as 1903 but as their procedure was difficult and cumbersome their method was never tried again

The introduction in 1922 by DOUGLAS and HALDANE of their rebreathing method provided with a fairly simple procedure for determining the CO_2 content of the mixed venous blood From this the amount of CO_2 eliminated and the CO_2 *content of the arterial blood* calculated from the CO_2 pressure in alveolar air the cardiac output could be calculated by the Fick equation

Rebreathing methods have since been widely used for cardiac output determinations for instance by BOCK, DILL and TALBOTT (1928) The technique was to rebreathe or hold in the lung different mixtures of CO in oxygen The rebreathing was repeated several times until a mixture was found the tension of which did not change on breath holding The obtained CO_2 tension was then said to be that of mixed venous blood This procedures involved repeated re breathing for 15 sec and took a considerable time

A method which made it possible to determine the mixed venous CO pressure from a series of rebreathings in and out of a bag and the breath by breath determination of the CO content of the bag was suggested by DEFARES (1956) The method to be described is founded on the above mentioned work by DEFARES and is based on the following assumptions

(1) No significant CO_2 pressure gradient exists between the end tidal air and the arterial blood

(2) The rate of blood flow during rebreathing is constant

(3) Recirculation of blood during rebreathing is negligible

During rebreathing from a bag the increase in CO content of the bag is dependent on (a) the lung bag volume (b) the blood flow through the lungs and (c) the CO_2 content of the mixed venous blood If the first two factors are constant the breath by breath increase of CO in the bag should follow an exponential course However the influence of the dead space in the lung bag system and the uneven distribution of inspired air in the lung can disturb the exponential relation of the first two or three CO_2 values

The above factors which have been subject to a very thorough mathematical treatment by DEFARES (1956) allow us to determine the CO pressure of the mixed venous blood and from that the cardiac output

Methods

The experiments are done in the morning with the subject in a fasting condition In the rest experiments the subject is lying almost horizontally in a resting chair The work experiments are done on a v DOBELN (1954) bicycle ergometer In the rest experiments the subject remains in the chair for about 30 min before the actual measurements are started After pulse control the subjects starts to breathe room air through a breathing valve with insignificant dead space (v DOBELN 1949) and the CO_2 percentage in the end tidal air is recorded with a rapid infrared CO_2 meter (Godart capnograph) The sensitivity of the CO_2 meter is adjusted with a calibration gas containing about 6 5 % CO_2 in air When the CO_2 concentration in end tidal air is stabilized the expired air is collected in a Douglas bag for 5 min during which the end tidal CO_2 is continuously registered with a potentiometer (Philips 1 sec for 90 % deflection) After the collecting is finished and the pulse rate again controlled the subject is allowed to rebreathe from a rubber bag at a constant speed This rubber bag is initially filled with about 2—3 % CO_2 30—40 % oxygen and nitrogen The amount of gas mixture in the bag during this rest experiment is about 1 liter and the rate of rebreathing is at rest 30 breaths a minute and during work 50 breaths a minute The tidal volumes and also th

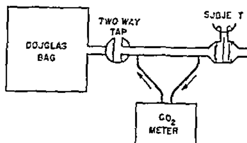


Fig 1 Arrangement for collecting air and measuring end tidal CO_2

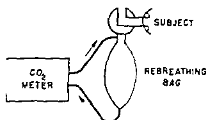


Fig 2 Arrangement for measuring CO_2 in lung bag system during rebreathing

breathing rate should be fairly constant The subject controls tidal volume by observing the size of the bag and breathing rate is governed by a metronome The CO_2 content of the bag is followed with the CO_2 meter which samples at a rate of 1 5 l/min The CO_2 meter is both during collection of expired air and during rebreathing coupled as a shunt to the breathing device Fig 1 and 2 show the experimental arrangements The volume of the tubes to the CO_2 meter analyzing cell and pump with connections is about 10 ml The subject rebreathes 10—11 times in and out of the bag After the re breathing which takes 12—20 sec is finished the subject is disconnected and the ex periment is for his part concluded

The contents of the Douglas bag are then measured in a wet gas meter and gas samples are taken for analysis with a Scholander apparatus At the same time a CO_2 meter reading is taken as an extra check on the CO_2 meter calibration

From the CO_2 percentage in the Douglas bag and the volume of the bag the CO_2 elimination is determined and from the O_2 values also obtained from the Scholander analyses the respiratory quotient and metabolic rate are obtained

The CO_2 percentages measured in the bag during rebreathing are now used for determining the mixed venous CO_2 content in the following way The percentage of the CO_2 in breath n is plotted against the CO_2 percentage of breath $n - 1$ in a linear coordinate system i e breath 2 against 1 3 against 2 4 against 3 and so on It is found that all the points obtained except the first one or two fall on a straight line

The distances between the plotted points are gradually decreasing and when the alveolar air and the mixed venous blood have the same CO_2 pressure the CO_2 percentage in two consecutive breaths should be the same This however never happens owing among other things to recirculation of CO_2 enriched blood The points where the CO_2 pressure in the bag and in the mixed venous blood is the same can however be extra polated from the straight line obtained This line is extended until it intersects a straight line with the same distance from ordinate and the abscissa The last mentioned line represents all the points where the CO_2 percentages in two following breaths are the same and the intersection of the two lines should thus represent the equilibrium point

Table I

Subject	Age years	Height cm	Weight kg	Body surface area m	No of determination	Cardiac output l/min	Cardiac output/m B.S.A.	Stroke volume ml	Basal metabolic rate
G L.	52	183	76	1.98	23	3.3	1.7	59	+ 5
L A	40	182	83	2.05	6	3.9	1.9	65	− 1
R J	33	172	60	1.72	28	4.0	2.3	50	+16
R L.	27	179	70	1.90	9	4.2	2.2	95	−11
D T	23	192	81	2.14	4	5.0	2.4	79	−10
K W	23	178	68	1.86	3	4.9	2.4	84	− 9
L L	18	177	72	1.89	5	3.9	2.1	70	−10
B B	17	185	79	2.05	3	4.9	2.4	89	−13

between CO pressure in alveolar air and blood i e the mixed venous blood Owing to the high oxygen percentages in the lung bag system this mixed venous CO_2 represents the CO_2 pressure in the oxygenated venous blood To determine the CO_2 content of the mixed venous blood and in end-capillary blood the data of ROOT in *Handbook of Respiration* 1958 are used The CO_2 capacity of the blood is assumed to be normal for all subjects

The cardiac output can now be calculated using Fick s equation viz

$$Q = V\ CO_2/(C_{\bar{v}CO_2} - C_{aCO_2})$$

where Q = blood flow per min in liters
$V\ CO_2$ = CO_2 eliminated per min in ml
$C_{\bar{v}CO}$ = CO_2 content of mixed venous blood in ml per liter of blood and Caco = CO_2 in arterial blood assumed to be the same as in end-capillary lung blood also in ml per 100 ml of blood

The subject were all healthy lung normal males of different ages The lungs were considered normal because a nitrogen elimination test showed normal ventilatory properties (LUNDIN 1955)

The physcial working capcity is normal for all subjects (ÅSTRAND 1960)

Results

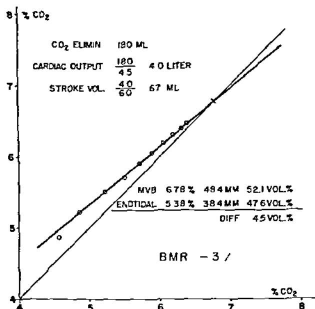
The first experiments were done at rest on subject G L to test the reproducibility of the method In a series of 23 exp the average cardiac output was 3 3 liters or 1 7 l/m² body surface area The standard deviation was 0 31 l and the coefficient of variation 9 4 % The average metabolic rate for G L during this series of experiments was + 5 %

In Table I the resting cardiac-output values for 8 subjects are summarized togehter with some anthropometric data All values where the CO_2 elimination per minute at rest is higher than 250 ml are excluded (usually the first 2—3 exp) The cardiac output seems to be smaller for the older subjects and is for the 5

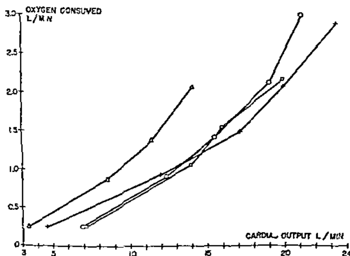
Table II

Subject	Work load kpm/min	No of determinations	CO_2 eliminated ml/min	A—V CO_2 difference ml/l	Cardiac output l/min	Stroke volume ml
G L	Rest	23	216	65	3 3	59
	300	3	[illegible]	[illegible]	8 5	97
	600	7	1 974	113	11 3	89
	900	2	2 005	143	14 0	85
R L	Rest	9	190	45	4 7	95
	300	2	742	76	9 8	101
	600	3	1 237	81	15 2	148
	900	2	1 770	97	18 2	154
	1 200	2	2 595	123	21 0	140
D T	Rest	4	215	43	5 0	79
	300	3	795	59	13 3	137
	600	4	1 400	61	23 1	191
	900	2	1 820	87	22 4	167
	1,200	2	2 920	115	25 3	146
K W	Rest	3	180	37	4 9	84
	300	2	775	74	10 5	118
	600	2	1 730	98	12 6	115
	900	[illegible]	1 675	110	15 2	118
L L	Rest	5	206	53	3 9	70
	300	5	854	60	14 2	137
	600	3	1 270	70	18 1	127
	900	1	1 910	84	22 7	134
B B	Rest	3	204	42	4 3	83
	300	2	804	70	11 6	121
	600	2	1 310	79	16 7	147
	900	2	2.270	109	20 7	138
Average						
R L	Rest	24	199	44	4 6	81
↓	300	14	[illegible]	68	11 9	125
	600	14	1,295	78	17 1	146
	900	9	1 890	98	19 8	141
P B	1,200	4	2 750	119	23 2	140

subjects under 30 years of age 2 3 l/m^2 body surface area The stroke volume too is smaller in the 3 older subjects than in the younger group being on average 58 ml against 83 ml Table II is a summary of the rest and work experiments o the 6 subjects on whom work experiments were done The increase in cardiac output during work is considerably higher in the 5 young subjects than in G L the increase in CO_2 eliminated is about the same for all subjects In G I



Fg 3 Graphical determina tion of CO in the mixed venous blood (MVB)



Fg 4 Relat onsh p between oxyg n consumpt on and cardiac ou put a rest and dur ng v ork

□ From Holmgren Jonsson and Sjostrand 1 60 D rect F ck method.
C From Asmussen and Nielsen 1953 Dye-d lution method
+ Our values from Table II Subjects R L to B B
– Our values from Table II subject G L.

the alveolar arterial CO_2 difference is considerably higher both at rest and during work than in the young subjects In all subjects there is a considerable increase of stroke volume from rest to work In subject G L the stroke volume during work seems to increase to a maximum already at 300 kpm where the stroke volume is 60 % higher than at rest and shows a falling tendency at higher work intensities whereas in the younger group the 600 and 900 kpm stroke volume values are on average higher than at 300 kpm/min with an increase over the resting value of about 80 %

Discussion

The cardiac output values obtained at rest are considerably lower than the now commonly accepted values obtained with the direct Fick or dye dilution methods but are of the same size as those obtained with the acetylene or nitrous oxide methods (GROLLMAN and BAUMAN 1935) This should stimulate researchers to undertake comparative simultaneous measurements of cardiac output with the CO_2 and for instance the dye dilution methods A comparison between the acetylene and dye dilution methods has been done by ASMUSSEN and NIELSEN (1953) who obtained a good agreement between the two method somewhat better during work than at rest The above mentioned authors conclude that both methods give reliable measures for cardiac output both at rest and during work However the resting values obtained by these workers in their combined measurements are considerably higher than the values obtained by them earlier with the acetylene method only The authors explain this partly by the fact that the earlier acetylene experiments at rest were done in a sitting position which gives somewhat lower values but they also attribute it partly to the apprehension caused by the experimental procedures (manipulation of the indwelling arterial needle etc) Another contributory factor could also be that the rest determinations were performed on each subjects only a few times and that they were therefore not in a condition quite as basal as were the highly skilled subjects used in the earlier acetylene experiments

From our experiments we can confirm the importance of the last mentioned factor In all our subjects the first one two or three resting cardiac output *values were higher than the later ones* and these higher values were also combined with a somewhat higher CO_2 output per minute For the 5 youngest subjects the average cardiac-output values for the first 2 measurements 6 0 liters or 3 0 l/m² instead of 4 6 l or 2 3 l/m² The average B M R found for these first 2 exp is + 15 % instead of an average of — 10 % in the experiments given in Table I

In Fig 2 a comparison is made of our values at rest and during work with those obtained by ASMUSSEN and NIELSEN (1953) using the dye dilution method and HOLMGREN JONSSON and SJÖSTRAND (1960) using the direct Fick method

The resting values from our experiments are considerably lower than those obtained by the above mentioned authors The values from the work experiments are about the same for our group of young subjects but lower for our oldest subject The main difference in results between our method and the dye-dilution and direct Fick method seems to be in the rest values We believe this to be due to the factor mentioned above the apprehension and higher metabolic rate for the methods in question If this is true the resting cardiac output should have about the same values as those given by GROLLMAN and BAUMANN (1935) viz around 2 3 l/m² body surface area higher for younger and lower for elderly subjects instead of the now generally accepted values of more than 3 l/m²

The decreased output with age is also in accordance with earlier observations where the acetylene method has been used (APERIA 1938) The considerable difference in stroke volume between rest and work confirm the older observations using the acetylen method (GROLLMAN and BAUMAN 1935) but does not conform with the values obtained by direct Fick (HOLMGREN JONSSON and SJOSTRAND 1960)

One of our assumtions on which the accuracy of the CO_2 method is dependent is that end tidal air and arterial blood have the same CO_2 pressure Direct measurements by EDWARDS and coworkers (1929) and SUSKIND *et al* (1950) seem to have confirmed that these pressures are the same both in reclining and in upright sitting subjects

A recent work by WEST (1962) using radioactive CO_2 to determine the ventilation perfusion ratio between different parts of the human lung seems to indicate a possible difference between end tidal CO and arterial blood of —1 mm Hg These measurements however are done during breath holding and this could influence the normal ventilation perfusion relationship If this pressure difference is accepted as normal it would introduce an error in our determination of the arterial blood CO_2 content of about 0 4 vol % which would give a theoretical error in cardiac output of less than 10 % This difference would also be reduced in the reclining position where the hydrostatic pressure difference which is supposed to cause these A — a CO_2 pressure differences is considerably decreased Our assumption that the CO_2 pressure in end tidal air has the same value as that of the mixed arterial blood thus seems justified for the resting experiments ASMUSSEN and NIELSEN 1956) found that during work there was a somewhat higher CO pressure in end tidal air than in arterial blood The same observation was made by SUSKIND *et al* (1950) who found that during work which gave about a 400 % increase in B M R there was a difference of about — 2 mm Hg between end tidal and arterial CO_2 pressure ASMUSSEN and NIELSEN found an increase in this difference with increased tidal volume beginning at tidal volumes of 1 4 l and amounting to about 3 mm during heavy work with tidal volumes around 3 l This could mean an error in our cardiac-output determinations during work consisting in values

around 5—10 % too high. Other possible sources of error in determination of the mixed venous blood are a change in lung volume during rebreathing and the recirculation of CO_2 enriched blood. DEFARES (1956) has shown that the changes in lung volume due to an oxygen uptake larger than the amount of carbon dioxide eliminated are too small to introduce any measurable errors. This should hold good at least for the rest experiments; during work however, there could be a decreased lung volume owing to a considerably higher amount of O_2 taken up during the later part of the rebreathing period tending to give gradually too high CO_2 values during rebreathing and thus a too high mixed venous blood CO_2 content. This error should counteract the above mentioned error resulting from the CO_2 pressure in end tidal air being higher than in arterial blood. However, the CO_2 error due to a volume decrease in the lung bag system is only of the order of a few hundredth of a per cent.

Recirculation could influence the CO_2 content in the last few breaths but from the curves obtained no tendencies are to be observed that indicate a deviation upwards of the plotted CO_2 curves. There is also during the first 3—4 rebreathing breaths some hyperventilation which means that the blood which passes through the lung during this short period leaves with a CO_2 content lower than normal. This blood should instead of a rising trend after 12—15 sec when recirculation is supposed to occur give a depressing effect in the exponential course of the CO_2 washing out and also counteract the above mentioned effect of the gradual increase of oxygen uptake versus carbon dioxide elimination. The CO_2 pressure of the oxygenated mixed venous blood should thus be determined with a fairly good accuracy. We thus have a method of determining the cardiac output in normal subjects which seems to be nicely reproducible, easy to perform and repeatable an infinite number of times. In subjects where ventilatory or circulatory disturbances makes it impossible to use the end tidal air to determine the arterial CO_2 pressure, it should still be possible to determine the mixed venous CO_2 pressure and thus avoid heart catheterization. This is of course only possible when the air mixing properties of the lungs during breathing are fairly normal. The method should also be ideal for following changes in normal subjects of cardiac output, stroke volume and a—v gas pressure differences due for instance to inactivity or physical training.

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Effects on Psychomotor Functions of Different Nitrogen-Oxygen Gas Mixtures at Increased Ambient Pressures

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Abstract

FRANKENHAEUSER M V GRAFF LONNEVIG and C M HESSER *Effects on psychomotor functions of different nitrogen-oxygen gas mixtures at increased ambient pressures* Acta physiol. scand 1963 59 400—409 — In order to differentiate and evaluate possible factors responsible for compressed air narcosis changes in psychomotor performance (simple and four-choice visual reaction times and mirror drawing) induced through exposure to different nitrogen-oxygen gas mixtures at raised barometric pressures were studied in 12 subjects By comparing data from five different experimental conditions evidence was obtained that at rest (1 nitrogen pressures up to 3 9 atmospheres absolute have but slight effects on objective performance and 2 oxygen excess has a potentiating effect on the narcotic action of nitrogen at high pressure It was concluded that oxygen excess acts indirectly by interfering with elimination of carbon dioxide from the tissues and that compressed air narcosis is not due to interference with oxidation in the tissues by nitrogen excess

Numerous investigations have shown that air at raised barometric pressures exerts an intoxicating or narcotic effect on man which is characterized by confusion euphoria slowed mental activity motor incoordination and impaired

performance Subjective intoxication has been observed at pressures low as 3 atmospheres absolute (atm), corresponding to a sea depth of 20 meters (BEHNKE THOMSON and MOTLEY 1935) whereas objective changes in performance have been demonstrated at 4 atm (KIESSLING and MAAG 1960) The true cause of these changes is still a matter of controversy Thus they have been attributed to a direct narcotic action of nitrogen at high pressure (BEHNKE THOMSON and MOTLEY 1935 CASE and HALDANE 1941 BENNETT and GLASS 1961), to the increased partial pressure of oxygen (DAMANT 1930) and to a buildup of carbon dioxide due either to hypoventilation (BUHLMANN 1961) or to increased difficulty in carbon dioxide movement in hyperbaric conditions (BEAN 1950) It has also been suggested that the subjective symptoms experienced in work at high atmospheric pressure are manifestations of anxiety (HILL and GREENWOOD 1906) and claustrophobia (see END 1938) or that their cause might be either a combination of all of the above mentioned factors or else an effect of the pressure alone (SHILLING and WILLGRUBE 1937)

The divergent opinion as to the cause of the narcotic action of air at high pressure may to some extent be explained by the fact that at raised barometric pressure there are simultaneous increases in alveolar oxygen pressure alveolar nitrogen pressure and gas density Experimental situations in which the subjects are exposed merely to a raised barometric air pressure will therefore not permit any differentiation between these factors as to their possible narcotic effects

Artificial gas mixtures containing less nitrogen and more oxygen than air have in recent years been employed in self contained diving apparatus because their use shortens the decompression time for a dive of given depth and duration It would therefore be of importance to ascertain whether such gas mixtures when supplied at high pressure produce narcotic effects in man and if so to make a quantitative comparison with the narcotic action of raised air pressure

In the present investigation an attempt was made to separate the possible factors causing compressed air narcosis by studying the changes in human performance induced through exposure to different nitrogen oxygen gas mixtures at elevated ambient pressures By comparing data obtained in five different experimental conditions the effects of 1) increasing the nitrogen pressure without changing oxygen pressure and 2) varying the oxygen pressure at a constant high level of nitrogen pressure were determined

Subjects and Methods

Twelve healthy subjects ranging in age from 19 to 39 years (average 26 1) were studied One of the subjects was a female technician three were professional male divers and the rest male medical students two of whom were amateur divers

Each subject was asked to perform three psychomotor tasks while breathing various oxygen nitrogen mixtures at normal or at increased ambient pressures in a dry compression chamber There were 5 experimental conditions (*Conditions A—F*) as follows

Condition	Ambient pressure atm	Inspired gas mixture	Partial pressures of inspired gases atm	
			Oxygen	Nitrogen
A	1.0	Air	0.20	0.74
B	1.0	100% O_2	0.94	—
C	4.2	5.2% O_2 in N_2	0.22	3.9[illegible]
D	5.0	Air	1.03	3.91
E	6.6	[illegible]9.8% O_2 in N_2	[illegible].60	3.94

The gas mixtures were administered by means of a respiratory system comprising a mouthpiece, a nose clip, a breathing valve assembly having a low resistance and small dead space (about 10 ml), and several Douglas bags placed inside the chamber. Each Douglas bag was connected to a high pressure cylinder outside the chamber. Visual and auditory communications were established between the observers outside and inside the chamber.

Psychomotor tasks

Four choice visual reaction time. The experimental arrangement used to provide the four choice task involved in this test has been described in full elsewhere (FRANKENHAEUSER, GRAFF-LONNEVIG and HESSER, 1960). The subject was seated before a panel on which were mounted, in triangular layout, three colored neon signal bulbs, a red below a green, and a yellow to the left of the two others. By means of an electronic device, including a magnetic tape recorder and tuned frequency sensitive relays, the three signals were switched on in random order and at random intervals which averaged 2 sec. The subject's task was to respond as rapidly as possible to the appearance of signals by closing one of two Morse keys according to the following scheme:

Red light — left hand key

Green light — right hand key

Red and yellow lights simultaneously — right hand key

Green and yellow lights simultaneously — left hand key

There was thus a four choice task involving reversals of response in addition to the basic two-choice task.

Each test trial included 40 responses, as follows. After one 10-sec period comprising 5 stimuli, there was a 5 sec pause during which the sum of the 5 preceding reaction times was read to 0.001 sec on an electronic decade counter. This procedure was repeated 8 times during a period of 2 min. For each subject the *score in reaction time* was calculated as the mean of his 40 reaction times. An inkwriter was used to record the signals and responses; these gave characteristic deflections, and in this way errors could be scored. The mean number of errors during each 2 min period gave the *error score* for each subject.

Simple visual reaction time. The technique and frequency of stimulus presentation were the same as in the choice reaction task except that one and the same stimulus (the yellow light) was invariably presented and the subject used one hand and one key only. The scores were the means of the 40 reaction times recorded within each 2 min period.

Mirror drawing. The task was to move a stylus along a track cut out in a metal plate so as to form a 5-pointed star, and visible only in a mirror. The track had saw tooth notches along both margins in which the stylus tended to get caught. The subject was

instructed not to touch the margins The time to complete one run was measured by a stop-watch and the time of contact between the stylus and the margins was recorded by the electronic decade counter Eight consecutive trials separated by 15 sec were given under each experimental condition. *Time score* was calculated as the mean time taken to complete the trials and *error score* as the mean time during which contact with the margins occurred

Experimental design

Each subject took part in two experimental sessions one of which comprised *Conditions A and B* the other *Conditions C D and E* Every other subject took the trials in *Conditions A and B* first. The conditions in the two sessions were rotated in the following manner

Subject 1	A B B A	C D E
Subject 2	D E C	A B B A
Subject 3	B A A B	E C D
Subject 4	C D E	B A A B
Subject 5	A B B A	D E C
etc		

In each condition the procedure was as follows

a) Simple reaction task 2 min
b) Mirror drawing 3 min
c) Choice reaction task 2 min

A 30-sec pause was inserted between successive tasks The two tests of reaction time were performed in the reverse order by half of the subjects

In the experiments under increased ambient pressures (*Conditions C—E*) the subject started to breath the assigned gas mixture while the pressure was changed from one level to another The rate of change in pressure was about 1 0 atm per minute The performance tasks were never begun until the subject had been breathing a new gas mixture for about 5 min and the desired pressure level had been held constant for at least 4 min Thus each subject spent about 12 min at each condition out of which 4 min were allotted to adaptation and 8 min to actual testing

The same procedure and schedule were used in the control experiments (*Conditions A and B*) except that normal atmospheric pressure prevailed throughout the experimental period

Those subjects who had not participated in previous experiments at increased ambient pressure and/or were not familiar with the psychomotor tasks were acquainted with the various aspects of the experimental situation at a preliminary session. Moreover to reduce effects of practice each experimental session was preceded by warming up trials in the three psychomotor tasks

Analysis of data

The data obtained in the 5 experimental conditions (*A—E* have been compared in the following ways

Comparisons I II and III By comparing *I*) *D* with *A* *II* *E* with *A* and (*III* *E* with *B* the effects of simultaneous increases in inspired P_{N_2} inspired P_{O_2} and gas density were determined

Comparisons IV and V By comparing (*IV*) *C* with *A* and (*V* *D* with *B* the effects of simultaneous increases in inspired P_{N_2} and gas density (with no change in inspired P_{O_2}) were estimated

Comparisons VI VII and VIII The effects of increasing the inspired P_{O_2} at a high P_{N_2} level (3 9 atm) were determined by comparing (*VI*) *D* with *C* (*VII*) *E* with *D* and (*VIII*) *E* with *C*

Table I Means and S E (12 subjects) for time and error scores in psychomotor tasks

Task	Condition				
	A	B	C	D	E
	Air (1.0 atm)	O_2 (1.0 atm)	5.2% O_2 in N_2 (4.2 atm)	Air (5.0 atm)	39.8% O_2 in N_2 (6.6 atm)
Simple reaction sec	0.243±0.005	0.242±0.006	0.241±0.007	0.248±0.007	0.256±0.006
Choice reaction sec	0.671±0.020	0.683±0.020	0.685±0.030	0.691±0.029	0.698±0.031
Choice reaction, number of errors	2.23±0.33	1.91±0.41	1.64±0.47	1.87±0.33	2.00±0.52
Mirror drawing time sec	9.16±0.67	9.25±0.70	9.47±0.64	9.24±0.59	8.93±0.49
Mirror drawing error sec	2.89±0.27	2.85±0.25	3.39±0.27	3.11±0.29	3.34±0.27

Table II Per cent changes in time and error scores in comparisons I—VIII Mean values for 12 subjects

Task	Comparison no							
	I	II	III	IV	V	VI	VII	VIII
	ΔP_{O_2} = +0.83 atm ΔP_{N_2} = +3.17 atm	ΔP_{O_2} = +2.40 atm ΔP_{N_2} = +3.20 atm	ΔP_{O_2} = +1.66 atm ΔP_{N_2} = +3.91 atm	ΔP_{O_2} = +0.07 atm ΔP_{N_2} = +3.18 atm	ΔP_{O_2} = +0.09 atm ΔP_{N_2} = +3.91 atm	ΔP_{O_2} = +0.81 atm ΔP_{N_2} = −0.01 atm	ΔP_{O_2} = +1.57 atm ΔP_{N_2} = +0.03 atm	ΔP_{O_2} = +2.38 atm ΔP_{N_2} = +0.02 atm
Simple reaction	+2.3	+5.4	−5.8	−0.8	+2.8	+3.1	+3.3	+6.2
Choice reaction	+3.0	−4.0	+2.1	+2.1	+1.2	−0.9	−0.9	+1.8
Mirror drawing (time)	+0.8	−2.5	−3.4	−3.3	−0.1	−2.4	−3.3	−5.7
Mirror drawing (error)	+7.6	−15.8	−17.3	−17.3	−9.1	−8.3	+7.6	−1.3

Significant at the 5 per cent level of confidence

The statistical significance of the observed differences was evaluated by submitting the intra pair mean differences between various conditions two at a time to the *t* test (see *e g* FISHER 1948

Results

The means of the twelve individual average scores for each of the variables under each condition are shown in Table I and the per cent changes in the scores in Table II *Comparisons I—VIII* see Subjects and Methods") In

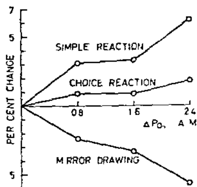


Fig 1 Changes in performance induced by increasing the oxygen pressure at a constant high nitrogen pressure (3 9 atm)
Mean values for 12 subjects

spection of the tables shows that there were only slight or moderate differences in performance between the conditions

Effects of increased P_O P_N and gas density A rise in air pressure from 1 0 to 5 0 atm caused only a slight tendency towards impaired performance (*Comparison I Table II*) However when a similar rise in inspired P_N was combined with a greater rise in inspired P_O (*Comparisons II and III*) a more pronounced and statistically significant impairment ($P < 0\ 05$) was induced in two of the performance variables (simple reaction time and mirror drawing error score)

Effects of increased P_N and gas density As revealed by *Comparisons II and I* a rise in inspired P_{N_2} (with no change in inspired P_O) produced but very slight changes in performance Only the error score in mirror drawing increased significantly ($P < 0\ 05$)

Effects of increased P_O at a constant high P_N At a constant high level of nitrogen pressure (3 9 atm) the simple and choice reaction times tended to increase with increasing oxygen pressure whereas the time score in mirror drawing showed a tendency to decrease (*Comparisons VI—I III*) Two of these changes reached a statistically significant level ($P < 0\ 05$) when the rise in P_O amounted to 2 4 atm (Fig 1)

In order to examine possible differences in intra individual variability between the five experimental conditions coefficients of variability based on the average individual variances were computed The coefficients obtained showed that there were no systematic differences in variability between the different conditions

Discussion

Our observation that air at 5 0 atm pressure produced only a slight impairment in objective performance (2—3 per cent increase in simple and four-choice visual reaction times) is at variance with those of SHILLING and GRUBE (1937) and KIESSLING and MAAG (1960) but agrees with

and HALDANE (1941) SHILLING and WILLGRUBE found a significant 10 per cent increment in light to touch reaction time at 5 5 atm pressure, and KIESSLING and MAAG a significant 21 per cent increment in two choice visual reaction time at 4 0 atm CASE and HALDANE observed only a somewhat intoxicating effect of air at 8 6 atm with no reduction of manual dexterity These quantitative deviations in results may possibly be due to pronounced individual differences in susceptibility to air narcosis or to differences in the general experimental procedure The observation of CASE and HALDANE (1941) that the narcotic effect of air at pressure is greatly enhanced by the addition of even minimal amounts of carbon dioxide to the inspired air indicates that the signs of subjective as well as objective intoxication in deep sea divers and in subjects breathing air in a compression chamber may in part be due to the carbon dioxide that accumulates in the diver s helmet or in the chamber In the present experiments special precautions were taken to prevent any admixture of expired carbon dioxide to the inspired air (see "Subjects and Methods)

Our data would then indicate that pure air when administered to resting human beings at 5 0 atm ambient pressure has only a very slight depressant effect on objective psychomotor performance This does not necessarily imply however that concomitant changes in subjective variables are also very slight In a previous investigation of the effects induced by various dose levels of nitrous oxide it was shown that performance in some tasks may remain relatively undisturbed when there is a pronounced increase in subjective intoxication (FRANKENHAEUSER and JARPE 1962) Similar observations were made in experiments dealing with effects of other drugs on various mental functions (FRANKENHAEUSER JARPE and MATELL 1961, FRANKENHAEUSER and POST 1962) The statement that air at 5 0 atm pressure produces only slight objective changes in performance is therefore not inconsistent with the observations of BEHNKE *et al* (1935) that subjective intoxication may occur at pressures as low as 3 0 atm

Since a rise in air pressure involves simultaneous increases in oxygen pressure nitrogen pressure and gas density *Comparison I* cannot be used to differentiate or evaluate the factors responsible for the performance changes observed at 5 0 atm The same holds for *Comparisons II and III* The fact that on raising the ambient pressure the impairment in performance became more pronounced if oxygen was added to the inspired air (*Comparison II*) indicates however that nitrogen and oxygen act in a synergistic rather than antagonistic fashion The conclusion may then be drawn that compressed air narcosis is not due to interference with oxidation in the tissues by nitrogen under high pressure

Support for the view that oxygen excess has a potentiating effect on compressed air narcosis is provided by *Comparisons II – III* which demonstrate that at a constant high P_N level (3 9 atm) the changes in performance increased with increasing oxygen pressure In a previous investigation it was found that on raising the inspired oxygen pressure to 3 0 atm at a low nitrogen pressure

there was only a slight tendency towards poorer performance (FRANKENHAEUSER *et al* 1960) The effects of oxygen thus seem to be more marked under conditions of high nitrogen pressure Our present data do not allow any definite conclusions as to the immediate cause of the observed differences in effects of oxygen It seems possible however, that oxygen excess may act indirectly by interfering with carbon dioxide elimination from the tissues This conclusion is based on the following observations LAMBERTSEN *et al* (1953 a b and c) found that the inhalation of 100 per cent oxygen at 3—3 5 atm caused an increased activity of the respiratory center due to local accumulation of carbon dioxide a 5—6 mm Hg fall in alveolar and arterial P_{CO} and a 3 mm Hg increment in internal jugular P_{CO_2} indicating that the CO_2 tension in the respiratory center had also increased by about 3 mm Hg To induce a similar rise in internal jugular P_{CO_2} by CO_2 breathing at normal atmospheric pressure the inspired CO tension had to be raised to about 20 mm Hg CASE and HALDANE (1941) found on the other hand that on increasing the inspired CO tension to 25—30 mm Hg the performance changes observed at 10 atm air pressure were markedly enhanced whereas at normal atmospheric pressure the same inspired CO_2 tension caused no deterioration in manual or arithmetical skill

From these and other results in the above mentioned investigations it may be inferred that (1) an increase in inspired P_{O_2} to higher than normal levels causes a rise in tissue P_{CO} (2) high N_2 and CO_2 tissue tensions have a synergistic narcotic action and (3) an isolated moderate increase in tissue P_{CO} has no demonstrable narcotic effect These three statements would seem to be compatible with our findings that at a high P_N level the performance changes increased with increased inspired P_O whereas at a low P_N level a similar increase in inspired P_O had only a small and insignificant effect on performance

As to the role of nitrogen in compressed air narcosis the present data (*Comparisons II and V*) permit the conclusion that at rest nitrogen pressures up to 3 9 atm have but very slight effects on objective performance Evidence for some impairment of judgement and or neuromuscular coordination was obtained from the error scores of the mirror-drawing task which increased significantly by 17 per cent when the nitrogen pressure was raised to 3 9 atm That nitrogen pressures below 4 0 atm have little if any narcotic effect on the respiratory center has been shown in earlier experiments on breath holding (HESSER 1962) in which evidence was obtained that the increased breath holding ability found at raised air pressure is not due to the increased P_N

BEAN (1950) and BUHLMANN 1961) maintain the view that the sole causative factor of compressed air narcosis is a rise in body CO tension Increased breathing resistance due to raised gas density at pressure would result according to BUHLMANN in hypoventilation and impaired CO elimination BEAN on the other hand contends that raised gas density leads to a reduction in CO diffusion and mixing in the alveoli and in this way to reduced CO output. Direct measurements on resting human subjects have shown however that after

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Acta physiol scand 1963 59 410—423

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Phasic and Static Excitability of Touch Receptors in Toad Skin

By

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Abstract

Lindblom U *Phasic and static excitability of touch receptors in toad skin* Acta physiol scand *1963 59 410—423* — The excitability of the toad s touch receptors was studied during slowly increasing and static deformation Graded mechanical stimuli of a linearly rising and a plateau phase were applied to single receptors *in situ* together with brief superimposed test pulses Action potentials were recorded from the afferent fiber in the dorsal root

In the moving stimulus phase the excitability rose rapidly and then displayed either a peak or a smooth flattening (Fig 4) As the plateau phase of the stimulation set in the excitability suddenly fell but a residue of raised excitability persisted statically (Fig 5 6) The less rapidly adapting receptors even presented a discharge in the plateau phase The impulse frequency of this discharge rose linearly with the deformation amplitude (Fig 9) but the maximum frequency was low as compared with that in the dynamic discharge While the static excitability change and the plateau discharge were related to the amplitude of deformation the dynamic excitability change like the dynamic discharge (Lindblom 1962) depended on the rate of deformation

An hypothesis that there may be two mechanisms — one phasic and one static — in one and the same receptor is discussed The course of the dynamic excitability explains the latency variations of the discharge following stimulation at varying deformation rates and may also account for the regular firing at constant deformation rate (Lindblom 1962) The characteristic rapid adaption is best explained as due to lack of excitation during static stimulation

In a recent series of papers (LINDBLOM 1958, HOGLUND and LINDBLOM 1961, LINDBLOM 1962) the excitability and impulse discharge of the toad's touch receptors were analyzed by means of mechanical stimulation of variable shape and strength. Common for all receptors were certain basic qualities such as their proneness to respond to dynamic stimuli and their rapid adaptation, but fairly large quantitative variations were observed, e. g. in rate of adaptation. Thus some receptors responded only during the initial, i. e. the moving phase of the stimulus, whereas in others a discharge — even though of limited duration — was obtained also during the subsequent plateau phase. The two receptor variants — termed "very rapidly adapting" and "less rapidly adapting" — could however not be classified as two separate types of receptors since various intermediary and transitional forms were observed. It does not seem unlikely, however, that there may be two mechanisms in one and the same receptor, one of which may account for the response to dynamic and one to static stimulation. More direct evidence in favour of this concept was obtained as the present investigation progressed.

The immediately preceding work (LINDBLOM 1962) described the characteristics of the dynamic discharge.[1] In the present investigation — performed on the same type of preparation — the plateau discharge has been studied more closely, special attention being paid to the relation between impulse frequency and amplitude of displacement. For a further analysis of the receptor properties and to be able to interpret the various discharge phenomena observed, it seemed necessary to know more about the excitation process in the receptor. Unfortunately, the preparation is not suitable for direct recordings from the receptor region, but adequate information could be obtained indirectly by studying the excitability changes accompanying subthreshold dynamic and static deformation, and most of the results described below were derived from such experiments.

Methods

Decerebrate and curarized Swedish toads (*Bufo bufo*) were used in all experiments which were performed at room temperature (20—23° C). Only receptors connected to coarse afferent fibers (A fibers, cf. LINDBLOM 1958, p. 29) and located on the plantar surface of the hindfoot or on the lower leg were studied. Results are based on recording from 115 receptors, of which 60 were classified as being of the very rapidly adapting and 55 of the less rapidly adapting type.

The performance of the mechanical stimulator (the same as used in two previous investigations, cf. HOGLUND and LINDBLOM 1961, LINDBLOM 1962) was as follows: rate of rise of the mechanical pulse continually variable between 0.08 and 80 mm/sec, maximum amplitude of displacement 300 μ. The brief mechanical pulse used for test stimula-

[1] In this paper the terms "dynamic" and "static" are employed to designate discharges, excitability changes etc. occurring in the rising phase and the plateau phase of stimulation, respectively. The terms "phasic" and "static" refer to the two mechanisms which may account for receptor responses of dynamic and static type respectively (cf. Discussion on page 420).

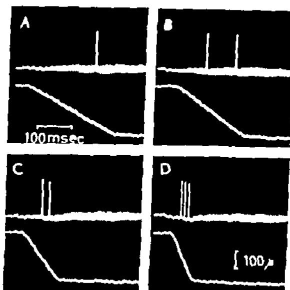


Fig. 1. Recordings from very rapidly adapting touch receptor (upper beam) and capacitance meter (lower beam) illustrating impulse discharge and effect of mechanical stimulation at progressive rate of deformation. A 1.0 mm/sec (= critical slope), B 1.3, C 2.3, D 3.8 mm/sec. Latency of first impulse in A—D 190, 85, 50 and 25 msec respectively, including calculated time (5 msec) of impulse conduction from receptor to recording site. Plateau amplitude of deformation 260 μ in all records. Rheobase value 20—30 μ (fluctuating, cf. LINDBLOM 1958, p. 25). From receptor on posterior part of planta pedis.

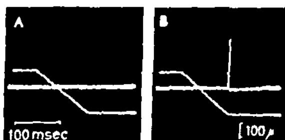


Fig. 2. Stimulation of very rapidly adapting receptor. A at slightly lower gradient than critical slope. B at critical slope (2.3 mm/sec). Plateau amplitude of deformation 300 μ. Rheobase value 60 μ. From receptor on planta pedis close by lateral pad.

tion in condition experiments was produced by means of a square wave of 1.5 msec duration from a grass stimulator connected to the loudspeaker of the mechanical stimulator. By slightly modifying the square wave by a condenser coupling oscillatory disturbances in the mechanical system could be avoided.

The mechanical stimulation was transmitted to the toad's skin via a small blunt rod so adjusted as to give an inward perpendicular displacement of the skin surface which was recorded by means of a capacitance meter coupled to the second beam of the oscillograph (for details see LINDBLOM 1958). All threshold determinations serving as basis for excitability curves were repeated two to four times, the points in the curves representing the mean values.

The impulse discharge from the receptor was led off from the afferent fiber in the dorsal root which was dissected into filaments thin enough to permit a study of single unit activity. Monopolar recordings of the action potentials were made from the end of the filament across a small air gap. The electronic recording equipment was of conventional type. A Tektronix oscilloscope type 502 (AC coupled) was used together with Grass amplifiers, stimulators and camera.

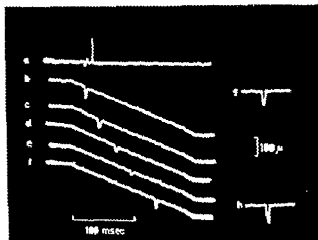


Fig 3 Excitability changes in very rapidly adapting receptor on slowly increasing deformation (rate 1 3 mm/sec just below critical slope) *a* impulse recording from afferent fibre *b*—*h* skin displacement Action potential in *a* elicited by test pulse which is seen as downward deflection superimposed on slow displacement stimulus (*b*) —*f* course of threshold as deformation progresses (corresponding dorsal root recordings not reproduced *g*—*h* threshold amplitude of test pulse alone before (*g*) and after (*h*) conditioning experiments intermediary values estimated by interpolation From receptor on planta pedis

Results

Dynamic excitability Fig 1 — shown in order to recall certain discharge phenomena described in earlier papers and referred to in the following — illustrates what happens when a touch receptor of the very rapidly adapting variant is stimulated at suprathreshold amplitude and progressive rate of displacement At the critical slope (ADRIAN CATTELL and HOAGLAND 1931 GRAY and MALCOLM 1951 GRAY and MATTHEWS 1951) a single impulse is elicited in the dynamic phase of stimulation after a fairly long latency A) As displacement rate is enhanced the latency gradually falls and new impulses are recruited B—D) Simultaneously there is an increase of the discharge frequency which was found to be related to the logarithm of the displacement rate Another typical feature of the dynamic discharge is that the impulse frequency is constant during a linearly rising stimulation i e when the rate of deformation is constant (LINDBLOM 1962)

In the first series of experiments to be described an investigation was made of the excitability changes occurring at a displacement gradient just below the critical slope (Fig 2 A) By using a subthreshold gradient as conditioning stimulus it was possible to avoid distortion of the curves due to the post spike refractoriness which is pronounced in the type of nerve endings under study (LINDBLOM 1958) As test stimulus a brief mechanical pulse was superimpo threshold amplitude of which was used as a measure of the excitability

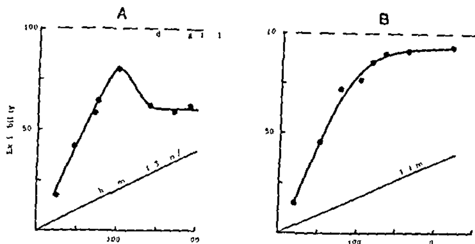


Fig 4 Curves illustrating the two types of excitability changes observed during slowly increasing deformation Note peak in A (from same experiment as Fig 3) and smooth flattening course in B (from another receptor at base of fourth digit critical slope in this case 2 5 mm/sec) Course of mechanical stimulation depicted below curves

Fig 3 illustrates how the stimulation was performed Before and after each conditioning series the threshold for discharge with the test stimulus alone was determined (g and h recepestively) The action potential in a is elicited by the test pulse which is recorded as a downward deflection superimposed after 2) msec, on the slow condition stimulus (b) The threshold amplitude of the test pulse is slightly lower than the unconditioned value in g As exactly measured the threshold fall — ie increase in excitability — amounts to 20 % The course of the threshold as the deformation progressed was followed by successively delaying the test pulse as shown by the stimulus recordings c –f (the corresponding dorsal root recordings are not reproduced) The threshold attained a minimum value about 100 msec after onset of the slow deformation (e) and then rose again (f)

The figure also illustrates one of the two types of excitability changes observed and the excitability course is depicted graphically in Fig 4 A in which the threshold fall in per cent of the unconditioned value is plotted against time from onset of the linearly rising stimulation Measurements of the kind shown in these figures were performed on fourteen receptors five of which exhibited the type of excitability curve depicted in Fig 4A ie they attained a maximum within the first 200 msec In the remaining nine receptors there was an initial equally fast increase in excitability to a high level but in contrast to the former group the excitability was maintained at this level or even showed a slight further increase during the whole course of linear deformation (Fig 4B and 6)

As a rule the excitability was tested only at a given gradient just below the critical slope, but in some experiments various lower gradients were followed

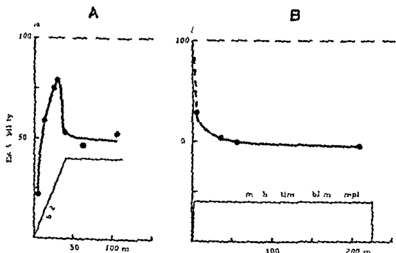


Fig 5 Excitability changes in two touch receptors illustrating rapid decrease of excitability as conditioning pulse (below curves) shifts from dynamic to plateau phase Note residual threshold reduction of ... from very rapidly adapting receptor on lateral pad (critical slope 8 mm/sec) B from somewhat less rapidly adapting receptor at base of fourth digit (critical slope around 1 5 mm/sec)

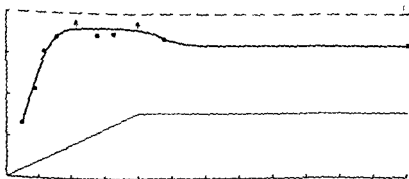


Fig 6 Excitability changes in very rapidly adapting touch receptor showing flattening of curve in dynamic phase and marked residue of raised excitability in plateau phase Arrows indicate values between 90 and 100 (amplitude of test pulse too small to permit exact determinations) From receptor at base of first digit (critical slope 1 5 mm/sec)

When starting at a gradient several times lower than the critical slope and increasing it stepwise there was a progressively steeper rise of the excitability up to increasingly higher levels until the receptor presented a discharge This occurred when the excitability reached the level marked discharge level in Fig 4, corresponding to 100 % threshold reduction The first impulse initiated either in the course of the slope as in Fig 1 A or at its end as

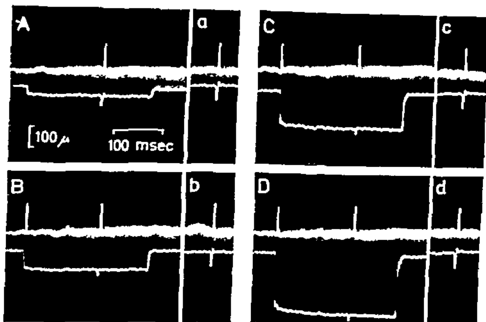


Fig 7 Effect of increased deformation on static excitability rise in very rapidly adapting receptor (same as Fig 6) Threshold amplitude of test pulse distinctly reduced even at subliminal (A) and slightly supraliminal (B) static deformation (rheobase value about 70 μ) No marked further threshold fall on increasing deformation (C D a—d threshold amplitude of test pulse alone after conditioning series

2B In the former case the excitability curve was of the type displaying a peak (Fig 4A) in the latter case it represented the type with a more or less smooth flattening course (Fig 4B)

Static excitability As mentioned above no discharge was obtained from *very rapidly adapting receptors* (such as all those illustrated in Fig 1—7) when exposed to static deformation even though the displacement amplitude amounted to several times the rheobase value Since some changes in excitability were anyhow likely to occur the threshold was followed also during the plateau phase of the stimulation Two typical results from such experiments appear from Fig 5 and 6

In the receptor of Fig 5A the high initial excitability immediately fell in the plateau phase so *that there was a threshold reduction of about 50 %* This excitability level was then maintained for the duration of the stimulation A similar level was observed in all receptors studied the reduction in threshold as a rule amounting to 50—80 % when the maximum displacement amplitude of the stimulator 300 μ was applied

The excitability level in the plateau phase remained remarkably steady for some length of time Thus the receptor of Fig 6 exhibited a threshold reduction by 80 % as long as after 400 msec of static stimulation As a rule a significant threshold reduction persisted even after several minutes constant dis-

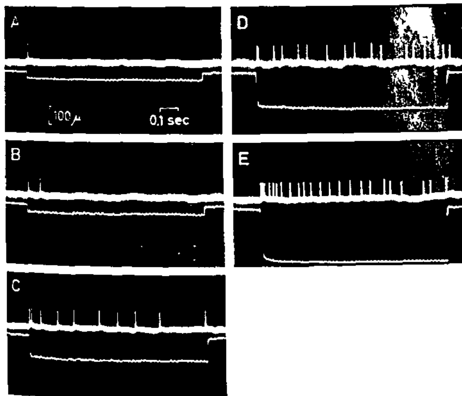


Fig 8 Plateau discharge in less rapidly adapting touch receptor at varying amplitudes of deformation Amplitude in *A* adjusted to threshold of first dynamic impulse that in *B* to the somewhat higher threshold of the first static impulse *C—D* increase of duration and impulse frequency of plateau discharge Although too frequent to be visible at sweep speed used impulses are recruited also in initial dynamic discharge From receptor on planta pedis Rheobase value 35 μ Critical slope < 0 08 mm/sec

placement of 100—300 μ a deformation which from a physiologic point of view is within moderate limits

The abrupt fall in excitability as soon as the stimulation changed from dynamic to static (Fig 5A) was further illustrated in experiments using a conditioning mechanical pulse of virtually rectangular shape (Fig 5B) With this type of stimulation an initial peak of dynamic excitability was obtained which could however not be traced for more than 30 msec at most after the static phase had set in The time constant for the fall of the dynamic excitability could not be exactly determined but was probably less than 10 msec In this latter type of experiments subliminal amplitude was used for the conditioning stimulation so as to avoid interference from the refractoriness after the action potential which would have given the curve a quite different course (virtually the reverse of the curve in Fig 5 B) The same type of experiments as illustrated in Fig 5 B has previously been done on the corresponding receptor in the

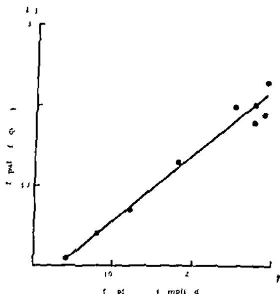


Fig. 9. Diagram of relation between impulse frequency of plateau discharge and displacement amplitude. Same experiment as Fig. 8.

frog's skin by GRAY and MALCOLM (1951) who found a constant threshold fall from 40 to 360 msec calculated from onset of the conditioning stimulation. With the stimulus amplitude used by them the reduction did not exceed 3.5 %. That a fall in threshold occurs even in the first 30 msec was also recently shown by CATTON (1962).

In order to study the significance of the stimulus amplitude for the degree of the threshold depression during static deformation experiments of the type shown in Fig. 7 were done. The test pulse was delayed about 150 msec from onset of the conditioning stimulation so as to avoid interference from the refractoriness after the initial impulse evoked by this stimulation at higher amplitudes (B—D). Even during subliminal static deformation as in record A the threshold was found to be significantly reduced (cf. the unconditioned test stimulation in *a*). In B the threshold is still lower. A further increase of the deformation amplitude did, however, hardly cause any further threshold fall (C—D). This means that the major part of the fall in threshold occurs at low deformation amplitudes below and the rheobase value.

Plateau discharge. The relation between impulse frequency and stimulation amplitude in the plateau discharge obtained in the *less rapidly adapting receptors* was analyzed as illustrated in Fig. 8. The stimulus amplitude in A is adjusted to the threshold of the first dynamic impulse and that in B to the slightly higher threshold of the first impulse of the plateau discharge. In C a low frequency plateau discharge is elicited of a duration of about two thirds of a sec. (The impulse to the right is probably an off response.) Further increase of the stimulus amplitude (D—I) resulted in a longer discharge and higher impulse

frequency Fig 9 shows the relation between the displacement amplitude and the average impulse frequency of the plateau discharge during the first sec (the initial dynamic discharge and the off response excluded) As seen, the relation is linear A similar increase in frequency was found in other receptors It should be noted however, that the effect of an increase of the amplitude was limited in sofar as the maximum frequency obtainable was remarkably low amounting to between 10 and 50 impulses per sec in most receptors The discharge was also often fairly irregular (Fig 8 D) and easily fatigued on repetitive stimulation These findings are contrary to the observations made on the dynamic discharge which is regular and attains high impulse frequencies (LINDBLOM 1962)

A certain increase of the impulse frequency and of the duration of the plateau discharge could be obtained if the skin was statically deformed before the stimulation set in by lowering the stimulator so that the skin surface was deflected 100—300 μ Such a preset displacement could sometimes provoke a plateau discharge in receptors primarily responding only to dynamic stimuli The dynamic discharge *per se* was however fairly unaffected by a preset displacement no significant changes being observed in 19 out of 31 ca es Some increase was observed in 6 and some reduction in 6 cases When the preset displacement exceeded 300 μ the result was either a reduction both of the dynamic and of the plateau discharge or a damping of the deformation caused by the under lying bone structures (mainly on digits) A stationary discharge was never obtained

Discussion

In the experiments using slowly increasing deformation the impulse discharge in the receptors was found to be preceded by a smooth increase in excitability varying with the velocity of the stimulus in the same manner as has been shown for the receptor potential in the Pacinian corpuscle (GRAY and SATO 1953) and in the frog s muscle spindle (KATZ 1950) The dynamic excitability curves ob tained in the present experiments are likely to reflect a corresponding depolari zation process in the toad s touch receptors

Two different types of dynamic excitability curves were observed one dis playing a peak and one with a smooth flattening course (Fig 4 A and B) As mentioned above these two variants of excitability curves explain why the first impulse may be initiated in the course of the deformation phase or only to wards its end The reduction in latency following stimulation at gradients above the critical slope (Fig 1) is an obvious consequence of the progressively faster rise in excitability with increasing rate deformation since this implies that the excitability attains the di charge level progressively earlier

It seems conceivable that the course of the dynamic excitability curve ex plain also another discharge phenomenon *viz* that the impulse interval is constant throughout the deformation phase when the rate of deformation is constant (LINDBLOM 1962) This is actually remarkable since a progressive non

linear rise in threshold — with a sharp initial increase and a flattening off — has been shown to occur on repetitive firing (LINDBLOM 1958 p 40) As discussed previously (LINDBLOM 1962 p 359), a prerequisite for a constant impulse interval is that the increase in threshold is counteracted by an excitatory effect increasing in the same manner It was thus particularly interesting to find that the increase in excitability did actually pursue a course of a rapid initial *rise and a subsequent flattening* There is a striking resemblance between the excitability curve in, *e g* Fig 4 B and the curve of the cumulative threshold rise previously published (LINDBLOM 1958 Fig 20 25)

The increase in excitability during static stimulation may at least in part be a purely mechanical effect the static stimulus producing a *preset deformation* of the tissues so that only a minute further deformation is necessary to cause discharge This concept does however not exclude the possibility that a depolarization state may be attained even during static stimulation thus being at least contributory to the change in excitability Merely an increase of the amplitude of the static stimulus did actually sometimes give rise to a plateau discharge even though of limited duration It is also known that a strong tangential stretch of the skin may cause a slowly adapting discharge (LOEWENSTEIN 1956)

The static excitability rise in the very rapidly adapting receptors as well as *the plateau discharge in the less rapidly adapting ones were both dependent on* the deformation amplitude In some cases a transition of the former type of receptor into the latter variant could be observed after preset displacement of the skin These observations suggest that the static excitability change and the plateau discharge may have a common origin and that there may be a quantitative rather than qualitative difference between the receptors capable of and those not capable of a plateau discharge (for further discussion cf HÖGLUND and LINDBLOM 1961 p 118 and LINDBLOM 1962 p 360) There were however striking differences between the static excitability change and the plateau discharge on the one hand *and the responses obtained in the dynamic stimulation* phase on the other hand The dynamic excitability changes was more dependent on the rate of rise of the stimulus than on its amplitude as was also the impulse frequency in the dynamic discharge (LINDBLOM 1962) Above a certain gradient the threshold of the dynamic discharge was low (HÖGLUND and LINDBLOM 1961) and the impulse frequency high and regular (LINDBLOM 1962) The plateau discharge had a higher threshold low maximum frequency and was often fairly irregular These differences suggest that two different receptor mechanisms — one phasic and one static — may be involved and a classification on this basis of the various phenomena observed may actually be more natural than the so far applied correlation solely to the dynamic and static type of stimulation This appears *e g* from an analysis of the impulse patterns obtained at extreme stimulus parameters Thus in the less rapidly adapting receptors a very slow *rate of deformation* gave rise to a discharge of static type even though the stimulation was dynamic whereas at very rapid rates the situation might be reversed

The discharge observed in the less rapidly adapting receptors at low deformation rates (LINDBLOM 1962 Fig 4 A) was of the same type as the plateau discharge The impulse frequency was on average low but was higher at the end at the beginning of the deformation phase This is consistent with the fact that the impulse frequency in the plateau discharge rises with increased deformation amplitude The discharge at low deformation rates may thus be conceived as being due to activation of the postulated static mechanism even though it occurred in the course of a dynamic stimulation This concept is supported by the fact that this discharge does actually correspond to that part of the slope frequency curve (LINDBLOM 1962 Fig 5) which is below the lower limit of the so-called optimal slope range in which the typical phasic properties of the receptors were apparent The very rapidly adapting receptors — whose static mechanism was weaker — did not present any discharge at all at these low deformation rates nor during the plateau phase of the stimulation Only at extremely high rates of deformation as at a virtually rectangular pulse an initial brief burst of impulses might sometimes be obtained in the plateau phase also in the very rapidly adapting receptors This discharge was of dynamic type the impulse frequency being high and not related to the stimulus amplitude and should thus be regarded as being due to a residual phasic excitation perhaps facilitated by subthreshold activation of the static mechanism (cf Fig 5 B)

If one presupposes a double mechanism in one and the same receptor it is tempting to make a comparison with slowly adapting mechanoreceptors As is well known especially as regards the muscle spindles (MATTHEWS 1931 KATZ 1950 JANSEN and MATTHEWS 1962) there is a dynamic and a static component also in their discharge Apart from possible dissimilarities in the accommodation of the supplying nerve fibers the rapidly and slowly adapting receptors may differ only insofar as the static mechanism may be more effective in the latter resulting in a lower threshold and a stationary discharge Also tactile units may display a slowly adapting discharge as shown in experiments on nerve skin preparations of frog (LOEWENSTEIN 1956) and on cat (HUNT and MCINTYRE 1960) but in general the phasic mechanism is predominant in touch receptors this is also consistent with their function being to record small rapid skin surface displacements For the preparation used in the present experiments the predominance of the phasic mechanism was pronounced and the plateau discharge when observed was relatively weak The static mechanism may thus be of less importance for the specific function of these receptors even though it may modify the phasic response

The observations above on the relation between amplitude of skin displacement and frequency of plateau discharge (Fig 9) may be compared with recent descriptions of the relation between muscle stretch and the adapted discharge in the crustacean stretch receptor (TERZUOLO and WASHIZU 1962) Although the type of deformation in the receptive nerve ending may be different the impulse frequency has anyhow in both cases been found to be directly proportional to the degree of deformation If the stimulus applied to the stretch receptor is expressed in terms of loading in grams instead of muscle stretch the result will be a logarithmic relation to the impulse frequency just as in the case of the mus-

cle spindles As far as the amphibian skin is concerned not much is so far known about the relation between loading and deformation amplitude

LOEWENSTEIN (1956) has proposed that the rapid adaptation in a mechanoreceptor may be due to the nerve endings recovering mechanically so that they are not activated during static stimulation This explanation has proved to be valid for the Pacinian corpuscle HUBBARD (1958) has shown that on application of a rectangular mechanical stimulation of the surface of the corpuscle, its center containing the receptive nerve ending undergoes only an initial deformation followed by an almost immediate mechanical recovery As far as the touch receptors in toad and frog skin are concerned, the anatomical conditions are different. The nerve endings are located superficially in the skin which is as a whole considerably deformed when displaced as in these cases several tenths of μ (LINDBLOM 1958 Fig 3) Under such conditions it does not seem likely that the endings should recover mechanically in the course of the stimulation unless this process may take place at the molecular level The characteristic rapid adaptation in these receptors should anyhow in the first place be interpreted as signalling absence of excitation This is the logical conclusion derived from the experimental results presented in the preceding papers as well as in the present investigation demonstrating that the receptors are activated solely or at least primarily by dynamic stimuli whereas static deformation is comparatively ineffective Some previous results and interpretations (DUN and FINLEY 1938 NAFE and KENSHALO 1958) are in keeping with this concept It may also be pointed out that static deformation within reasonable limits does not block the ability of the receptors to respond to dynamic stimuli This ability is maintained even after total adaptation of the discharge as shown by the occurrence of off responses and of a second burst of impulses on stepwise stimulation (NAFE and KENSHALO 1958 Fig 6) Nor was any appreciable change of the dynamic response observed in the present experiments after preset static displacement of the skin and LOEWENSTEIN (1956 p 598) has also found that tangential stretch of the skin may be applied without causing any marked change of the tactile response

If the concept outlined above holds true there is no need to assume an active counterprocess such as accommodation in the nerve fiber cf GRANIT and SKOGLUND 1943 KATZ 1950 GRAY and MATTHEWS 1951) as being essential for the adaptation in the type of receptors studied Such processes may anyhow significantly modify the discharge evoked The accommodation process may e g cause the flattening of the dynamic excitability curve and a damping of the plateau discharge

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Decerebrate Control of Reflexes to Primary Afferents

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Abstract

Carpenter, D I Engberg H Funkenstein and A. Lundberg *Decerebrate control of reflexes to primary afferents* Acta physiol scand 1963 59 424—437 — Dorsal root potentials (DRP) have been compared in decerebrate cats before and after transection of the spinal cord The DRPs evoked from group I muscle afferents of flexor and extensor muscle are either unchanged or slightly decreased after transection of the cord In the spinal state the DRP evoked from cutaneous afferents has two components 1) Component I representing primary afferent depolarization (PAD) in cutaneous afferents has brief latency and is of equal size in the decerebrate and spinal states 2) Component II and the DRPs evoked from high threshold muscle and joint afferents are ascribed to the flexor reflex afferents (FRA) and represent PAD in the FRA This action cannot be evoked in the decerebrate state The FRA act on the same primary cutaneous afferents in which component I is evoked It is concluded that there is a tonic inhibition in the decerebrate state of transmission from the FRA to the FRA but there is neither inhibition of the short latency path from cutaneous afferents to cutaneous afferents nor of the paths from group I muscle afferents to Ia, Ib and cutaneous afferents The cord dorsum potentials (CDP) have also been compared before and after transection of the cord The release after brain stem lesions of the CDPs and DRPs evoked from the FRA is compared with the release of actions from the FRA to motoneurones

In decerebrate cats there is a tonic descending inhibition of transmission from the flexor reflex afferents (FRA) to motoneurones and to ascending pathways (Eccles and Lundberg 1959 b Holmqvist and Lundberg 1959 1961 Holmqvist Lundberg and Oscarsson 1960 Kuno and Perl 1960) In the

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present investigation dorsal root potentials (DRP) and cord dorsum potentials have been compared in the decerebrate and spinal state the purpose being to investigate decerebrate control of reflex paths to primary afferents There is a marked decerebrated inhibition of transmission from the FRA to primary afferents

Methods

The experiments with DRP recording were made on unanaesthetized decerebrate cats Ether was used during the operation and administered in suitable mixture with air and oxygen to give a constant depth of anaesthesia Recording was started 2 hours after withdrawal of ether In the experiments with intracellular recording from primary afferents the anaesthesia was chloralose urethane During the experiments the animals were immobilized with Flaxedil and artificially respirated The blood pressure was always measured and Dextran given if required

For recording DRPs a filament of the dorsal root was sectioned dissected free to the point of entry into the cord and mounted on recording electrodes one close to the spinal cord and the other at the cut end The interelectrode distance was about 1 5 cm. When effects were compared at different segmental levels care was taken to select filaments of equal thickness The cord dorsum potentials were recorded with one electrode placed on the dorsal root entry zone and the indifferent electrode in the surrounding muscle The time constant of the recording amplifiers was 0 8 sec The ventral roots L6—S1 were sectioned In all experiments the effect of conditioning volleys in the FRA on monosynaptic test reflexes was investigated before and after transection of the spinal cord All results reported were obtained from preparations in which before transection of the cord single volleys in the FRA did not evoke actions in motoneurones

Brain stem lesions were made as described by HOLMQVIST and LUNDBERG (1961) The spinalizations were made at the lower thoracic level

Abbreviations are for muscle nerves posterior biceps-semitendinosus, PBSt gastrocnemius-soleus G—S deep peroneal DP For cutaneous nerves superficial peroneal SP sural Sur For the posterior nerve to the knee joint J

Results

DRPs in decerebrate and spinal cats

Group I muscle afferents Volleys in group I muscle afferents evoke large DRPs in decerebrate preparations in which there is complete suppression of transmission from the FRA to motoneurones and to primary afferents (cf below) After transection of the spinal cord the group I DRP is either unchanged or slightly decreased as in Fig 1 in which a group I train was given in the nerve to PBSt The same finding was made with the DRP evoked by group I volleys from extensor muscles (G S or AbSm) ECCLES ECCLES and MAGNI (1961) have shown that Ia and Ib volleys contributed to presynaptic inhibition in the Ia pathway to motoneurones Likewise these two fibre systems contribute to the cord dorsum positivity evoked by group I train from flexor muscles (ECCLES, MAGNI and WILLIS 1962) We have confirmed this finding with respect to the DRP evoked by group I volleys from flexor muscles In the experiment of F

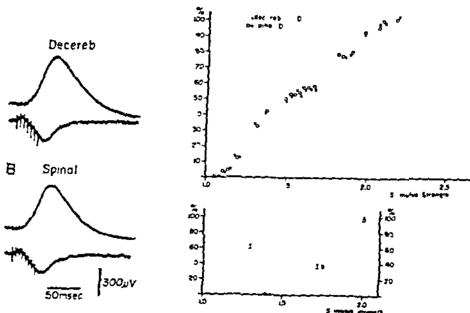


Fig 1 The upper traces in A and B are dorsal root potentials (DRP) recorded from the most caudal dorsal root filament in L6 in a decerebrate cat. The lower traces were recorded from the dorsal root entry zone in L7 Both records show the effect of a train of 6 group I volleys in the nerve to PBSt. Record A was obtained before and B after transection of the spinal cord In the DRP recording an upward deflection signals negativity of the electrode close to the spinal cord In the lower traces an upward deflection signals negativity of the electrode in contact with the cord dorsum. Calibration refers to the DRP

In the upper graph 100 % on the ordinate represents the maximal DRP evoked by the train of 6 maximal group I volleys in the nerve from PBSt. The group I volley from PBSt displayed complete separation in Ia and Ib volleys. The height of the DRP is plotted as a function of the stimulus stength in multiples of threshold strength before (x) and after (o) transection of the spinal cord. The lower graph shows the size of the Ia (x) and Ib (o) spike potential plotted against stimulus strength. Observe that for the Ia volley 100 % of the ordinate represents the height of the spike at the stimulus strength just activating all Ia fibres The increase above 100 is due to synchronization. Single stimuli were used for the lower curves The scale of the abscissa is identical in the upper and lower curves.

I the group I volley from PBSt displayed complete separation in Ia and Ib components The curves illustrate that more than half of the DRP can be attributed to Ia afferents There is no significant difference between the effect of Ia and Ib volleys obtained before and after transection of the spinal cord The positive cord dorsum potential (P wave) that is associated with the DRP (BARRON and MATTHEWS 1938 ECCLES MAGNI and WILLIS 1962) is of the same size before and after transection of the cord (Fig 1)

Group II and III muscle afferents Effects from high threshold muscle afferents to primary afferents are very effectively inhibited in the decerebrate state The records in Fig 2 illustrate effects evoked by single volleys in high threshold muscle afferents before and after spinal transection In the decerebrate state a maximal group I volley evoked a small DRP and there was very little additional

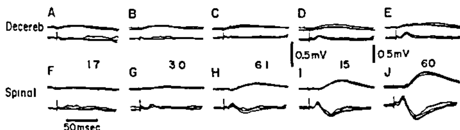
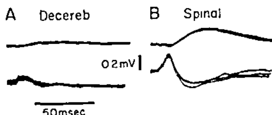


Fig 2 As in Fig 1 but showing the effect of single stimulus in the nerve to G—S before and after spinal transection Corresponding records in the upper and lower row were obtained at the same stimulus strength which is indicated between the corresponding records and expressed in multiple of threshold strength Calibration between record C and D refers to the DRP and calibration between D and E to the cord dorsum recording The records consist of superimposed oscillographic traces

Fig 3 As in Fig 1 but stimulation of the posterior joint nerve before and after transection of the cord The stimulus strength was about 20 times threshold for the nerve Calibration refers to the DRP recording The records consist of superimposed traces



effect when the stimulus strength was raised to 60 times threshold (Fig 2 E) in order to activate group III muscle afferents In the spinal state however a DRP and the associated P wave can be evoked from group II and III muscle afferents (cf BERNHARD 1953 ECCLES KOSTYUK and SCHMIDT 1962a) as is shown in G J

A comparison of corresponding upper and lower records also reveals that there is a release of the negative cord dorsum potentials evoked from high threshold muscle afferents In the spinal state they appear at lower threshold and are larger than in the decerebrate state In some decerebrate preparations group III volleys evoke only very small negative cord dorsum potentials (Fig 8 record J)

Joint afferents High threshold joint afferents activated at a minimal strength of 2 5 times threshold for the nerve belong to the FRA (ECCLES and LUNDBERG 1959a) Volleys in these afferents evoked DRPs and P waves in the spinal state (Fig 3 B) but are ineffective in the decerebrate state (A) The early part of the negative cord dorsum potential in A (Fig 3) is evoked from low threshold joint afferents (SKOGLUND 1956) This effect of low threshold joint afferents is illustrated in Fig 4 in which the cord dorsum potential (lower traces) were recorded at higher amplification in a decerebrate preparation The posterior knee joint nerve was stimulated at increasing strength of stimulation and volley was recorded from the sciatic nerve A threshold negative cord d

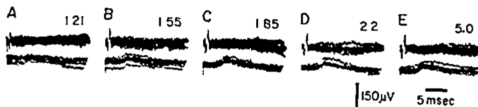


Fig 4 The lower traces were recorded from the cord dorsum at the L7 entry zone and the upper traces are triphasic recordings from the sciatic nerve The posterior joint nerve was stimulated at increasing strength indicated in multiples of threshold strength in each record Observe that the negative cord dorsum potential appears in A and does not increase when the stimulus strength is raised above 1 85 times threshold Calibration refers to the cord dorsum recording The records consists of superimposed traces

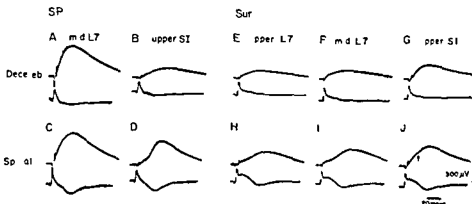


Fig 5 A—D As in Fig 1 but stimulation of the cutaneous SI nerve before (A B) and after (C, D) transection of the spinal cord The DRPs in A and C were recorded from a dorsal root filament in mid L7 and those in B and C from the most proximal filament in SI The stimulus strength was 3 1 times threshold for the nerve In all records the cord dorsum potentials were recorded from the dorsal root entry zone in L7 The records consist of superimposed traces E—J As above but recording from 3 filaments at the levels indicated and stimulation of the sural nerve at a strength of 13 times threshold for the nerve The maximal entry of sural afferents into the cord was through the lower filament in L7 Arrow in record J indicates the onset of the slow wave which is seen more clearly in records H and I All records consist of superimposed traces

potential is evoked in A at a strength of 1 21 times threshold and it does not increase when the stimulus strength is raised above 1 85 There has so far been no clear evidence that impulses in low threshold joint afferents give actions in motoneurones or to ascending spinal pathways (outside the dorsal column) but the finding that a negative cord dorsum potential can be evoked from them does indicate a synaptic relay in the dorsal horn

Cutaneous afferents A more complex situation prevails with respect to the actions from cutaneous afferents Some effects are identical in the decerebrate and spinal states but other effects are suppressed in the decerebrate state and appear only after transection of the cord

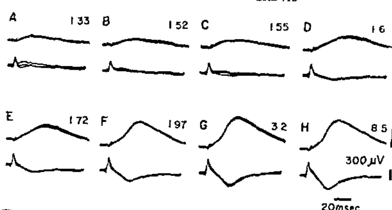


Fig 6 *The DRPs were recorded in a spinal preparation from the most proximal dorsal root filament in S1 The cord dorsum recording was from the entry zone in L7 The SP nerve was stimulated at increasing strength and cated in each record in multiples of threshold strength All records consist of superimposed traces*

The records A—D in Fig 5 were obtained on stimulation of the superficial peroneal (SP) nerve A and B were recorded in the decerebrate and C and D in the spinal state The left records were recorded from a dorsal root filament in mid L7 close to the entry zone of the afferents from the SP nerve and there is little differences before and after transection of the cord Records B and D in Fig 5 were recorded from the most rostral dorsal root filament of S1 The initial part of the DRP is identical in the two records but in the spinal state a late wave has been released The DRP which is identical before and after transection of the cord will be called component I It is evoked exclusively by low threshold cutaneous afferents the effect is maximal at 2—4 times threshold for the nerve (ECCLES KOSTYUK and SCHMIDT 1962a) Component I has a restricted longitudinal distribution in B half a segment from the zone of entry the size is about one third of the maximal DRP In filaments more than one segment away from the entry zone hardly any component I can be evoked

The slow wave which has a wider distribution along the cord and is released after transection of the cord will be denoted component II In Fig 5 (records A and C) it should be noted that component II does not appear in the filament of mid L7 in which a large component I is evoked Since component II can be evoked from rather low threshold cutaneous afferents (cf below) it is unlikely that this absence of component II is due to primary afferent depolarization caused by the fast fibres responsible for component I in the fibres responsible for component II A more likely explanation is that component I and II are evoked largely in the same primary afferents If a smaller cutaneous nerve is used for stimulation component II may appear superimposed on component I as is shown in Fig 5 records E—J in which the sural nerve was stimulated The majority of the sural afferents were in the lowest part of L7 and record was made from dorsal root filaments at the levels indicated in the Fig T

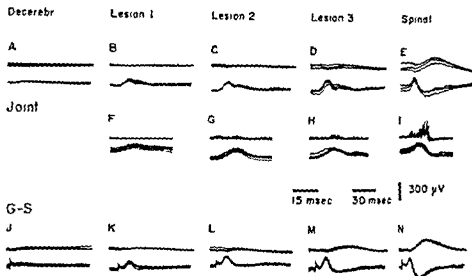


Fig. 8. Release of DRPs and cord dorsum potentials after brain stem lesions. Lower traces are from the cord dorsum in L7. Upper traces in A—E and J—N are from a dorsal root filament in lower L6 and in F—I from the S1 ventral root. The experiment was made on a decerebrate decerebellectomized cat and the records in each column were taken after the indicated lesions which are shown in the drawing of Fig. 9. In A—I the joint nerve was stimulated at a strength of about 25 times threshold. In J—N the G—S nerve was stimulated at a strength of 19 times threshold. Record F—I with ventral root recordings were taken at the faster sweep speed. It should bee noted that after lesion 2 the negative cord dorsum potential is slightly larger in G than in C. Record C was taken a few minutes after the lesion, record G 15 minutes later just before the corresponding curve in Fig. 9. Voltage calibration refers to the DRP. All records consist of superimposed traces.

Effect of brain stem lesions

The centres responsible for the tonic control of transmission from the FRA to motoneurones are located in the medial brain stem (HOLMQVIST and LUNDBERG 1959). On investigating the release after lesions at different brain stem levels it was found that inhibitory and excitatory paths to motoneurones are differentially controlled. A low pontine lesion releases the inhibitory path but a more caudal medullary lesion is required to release the excitatory paths (HOLMQVIST and LUNDBERG 1961, HOLMQVIST 1961). We have now compared the release of action to primary afferents from the FRA with the release of paths to motoneurones. In Fig. 8 (records A—I) the posterior joint nerve was stimulated and recording was made from a dorsal root filament in A—E and (at faster speed) from the ventral S 1 root. The lower traces are from the cord dorsum. The records were obtained after the lesions indicated in the drawing of Fig. 9. For comparison the curves in Fig. 9 show the effect after these lesions of the same conditioning volleys in the joint nerve on the monosynaptic test reflex from PBSt and G—S. With lesion 1 there is release of inhibition to G—S (Fig. 9) and of a negative cord dorsum potential (Fig. 8 B and F). Lesion 2 gives further release of these actions (C and G in the Fig.)

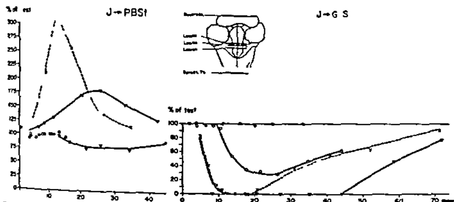


Fig 9 The effect of conditioning volley in the posterior joint nerve on the monosynaptic test reflexes from the nerve to PBSt (left graph) and the nerve to G—S (right graph) 100 of the ordinate represents the unconditioned amplitude of the test reflex Conditioned amplitude expressed as percentage on controlled amplitude is plotted as a function of time interval between conditioning and testing stimulation The measurements were obtained after the transverse medial brain stem lesions indicated in the drawing Same experiment as Fig 8

without any release of excitatory action to PBSt (Fig 9) Following lesion 3 there is some release of excitatory action to PBSt but there is no DRP (D Fig 8) Following transection of the spinal cord there is further release of excitatory action to PBSt (cf also ventral root records G—I Fig 8) and now the volley in the joint nerve also evokes a DRP and the associated P wave is recorded from the cord dorsum (E and I) A close inspection of records G—I reveals that the rate of rise and height of the negative cord dorsum potentials is indentical in the three records

Similar findings were made with the corresponding actions from high threshold muscle afferent and with component II of the DRP evoked from cutaneous afferents In the records J—N of fig 8 from the same experiment the G—S nerve was stimulated at a strength of 19 times threshold after the lesions indicated The effect of volleys in high threshold muscle afferents on monosynaptic reflexes from the PBSt and G—S nerves were investigated at the same time The differential release was very similar to that shown in Fig 9 for the effects from high threshold joint afferents and is not illustrated With the release of the inhibitory path with lesion 1 and 2 negative cord dorsum appeared (K and L) but hardly any DRP or P wave After lesion 3 which gave a partial release of the excitatory path to flexor motoneurones volleys in group III afferents evoke a DRP and the associated P wave (M) and after spinal section there is a further increase (N)

Discussion

The DRPs evoked by volleys in group I muscle afferents are either identical in the decerebrate and spinal states or else there is a slight decrease following transection of the spinal cord This holds true for effects from nerves of extensor

as well as of flexor muscles and from the latter both for the DRPs evoked from group Ia and Ib afferents Group I volleys are known to evoke primary afferent depolarization in Ia, Ib and in cutaneous afferents (ECCLES MAGNI and WILLIS 1962a ECCLES, SCHMIDT and WILLIS 1963 ECCLES KOSTYUK and SCHMIDT 1962, CARPENTER et al 1963) On the basis of the present findings it is suggested that there is no tonic decerebrate inhibition of the reflex arcs from group I to these different systems There is previous evidence that the pre synaptic inhibition of Ia pathway to motoneurones (cf FRANK and FUORTES 1957 ECCLES ECCLES and MAGNI 1961) is identical in the decerebrate and spinal states (KUNO and PERL 1960, cf HOLMQVIST and LUNDBERG 1961)

There is on the other hand clear evidence of tonic inhibition of the DRPs evoked from the FRA but before discussing this effect it is necessary to consider the finding that the DPR evoked from cutaneous afferents has two components Component I is evoked by low threshold afferents and has a brief latency (central delay 2 msec) The distribution of this component of the DRP along the cord is restricted An incoming cutaneous volley causes a large component I only in filaments adjacent to its entry zone One segment caudal or rostral to this zone the magnitude of component I is less than 25 per cent of the maximal one Component II is on the other hand evoked by low and high threshold cutaneous afferents and appears after a central delay of about 10—15 msec This component of the DRP has a wide distribution along the cord it can be recorded several segments from the entry zone Those findings confirm BERNHARD's (1953) results regarding cord dorsum potentials He found that the smooth positive deflection following the N_1 wave has a restricted longitudinal distribution very similar to that of the N_1 wave On the other hand for the deep positivity which followed the second negative wave (N_2) he found a much wider longitudinal distribution as has now been found for component II of the DRP In our experiments a stimulus strength of at least 1 5 times threshold was required to evoke component II whereas component I appears at a just supraliminal strength However BERNHARD (1953 Fig 13) found that the deep P wave above identified with component II of the cutaneous DRP could be obtained at very low strength of stimulation although usually at a slightly higher strength than that required to evoke the N_1 deflection

Of the DRPs evoked from cutaneous afferents only component II should be described as an FRA effect With respect to latency and distribution along the cord component II resembles the DRPs evoked from high threshold muscle and joint afferents Further evidence is provided by the decerebrate control of the paths responsible for these actions There is no evidence of decerebrate inhibition of component I but marked decerebrate inhibition of component II as well as of the effects from high threshold muscle or joint afferents We have confirmed that a large primary afferent depolarization which corresponds to component I of the DRP is evoked in cutaneous afferents by volleys in cutaneous nerves (KOKETSU 1956 ECCLES and KRNJVIC 1959) The experiments with DRP

recording suggest that components I and II are evoked largely in the same primary afferents and with intracellular recording this was found to be so in large cutaneous afferents It can therefore be concluded that in addition to the group I activated path discussed above there are two other reflex paths to primary cutaneous afferents One of them is supplied exclusively from low threshold cutaneous afferents of a restricted receptive field and there is probably only two interneurones in this pathway The other path is supplied from the FRA (including cutaneous afferents) from a wider receptive field and to judge from the latencies this pathway has a long internuncial chain The latter pathway but not the former is subject to strong decerebrate inhibition and the same must hold true for the effects from the FRA to high threshold muscle and joint afferents (cf ECCLES KOSTYUK and SCHMIDT 1962b) Further experiments are required to determine whether component I is evoked only in cutaneous afferents

Component II of the cutaneous DRP seems to be associated with the N_1 cord dorsum potential Following transection of the cord there is a large increase of this N_1 potential as well as of the negative cord dorsum potential from high threshold muscle and joint afferents It is assumed that these negative cord dorsum potentials represent excitatory postsynaptic potentials in interneurones and cells of ascending pathways) but presumably not to any large extent in second order neurones The FRA presumably have connection with relatively few second order neurones which activate many third order neurones The N_1 potential on the other hand presumably represents the monosynaptic excitatory postsynaptic potential in secondary neurones Previously LINDBLOM and OTTOSSON 1953 have reported an increase of the N_1 potential following transection of the cord GASSER and GRAHAM (1933) found that the N_1 potential was decreased for the duration of the positive cord dorsum potential which now has been associated with primary afferent depolarization and presynaptic inhibition ECCLES KOSTYUK and SCHMIDT 1962a) An increase of the N_1 potential following cord transection could have indicated removal of a tonic descending primary afferent depolarization However in our experiments the N_1 potential was the same in the decerebrate and spinal state and it is particularly relevant that the rate of rise of the potential was the same

Regarding the mechanism of decerebrate inhibitory control of transmission from the FRA ECCLES and LUNDBERG (1959 b) postulated an inhibition at an interneuronal level but there is also the possibility of descending inhibition through depolarization of primary afferents (ECCLES 1961) The centres responsible for the tonic descending inhibition in the decerebrate state are located in the ventral part of the medial brain stem Stimulation of this region does evoke primary afferent depolarization in the FRA (CARPENTER ENGBERG and LUNDBERG 1962)

The present experiments are only indicative with regard to the mechanism of decerebrate control It can be concluded that there is no tonic depolarization

in the decerebrate state of the cutaneous afferents responsible for component I of the DRP The same holds true for the low threshold cutaneous afferents from which the N_1 cord dorsum potential is evoked It is probable that the same holds true for the FRA but even if component I is evoked in fibres, which are activated upon by the FRA, the possibility that the low threshold cutaneous afferents of the FRA are other afferents than those referred to above cannot at present be excluded

It is also necessary to consider the finding of a differential control of actions from the FRA A low pontine lesion gives an almost complete release of the inhibitory path to extensor motor nuclei from decerebrate control without any release of the excitatory path to flexor nuclei which is released only after a more caudal lesion (HOLMQVIST and LUNDBERG 1961) The release of actions from the FRA to primary afferent parallels the release of the excitatory path to flexor motoneurones in its occurrence with a caudal medullary lesion (Fig 8 and 9) It is of interest that the negative cord dorsum potential is released by a low pontine lesion With the more caudal medullary lesion required for release of the DRP and the associated P wave there is no increase of the initial phase of the negative cord dorsum potential (Fig 8) This suggests that the synaptic actions exerted by the primary afferents is not increased by the latter lesion and hence that after a low pontine lesion the control of transmission (to primary afferents and of excitation to flexor motor nuclei) is exerted at an interneuronal level Subsequently it has been demonstrated that a stimulation of the medullary brain stem that does not evoke primary afferent depolarization can give marked inhibition of reflex paths to primary afferents (LUNDBERG and VYKLICKY 1963)

This investigation was supported by grants from the Swedish Medical Research Council

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The Effect of Cold Exposure on the Catecholamine Excretion of Adrenalectomized Rats Treated with Reserpine

By

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Abstract

JOHNSON G E *The effect of cold exposure on the catecholamine excretion of adrenalectomized rats treated with reserpine* Acta physiol scand 1963 59 438—444 — Adrenalectomized rats subjected to moderate cold exposure showed a significant increase in noradrenaline excretion in urine Adrenalectomized rats treated with reserpine however failed to respond with an increase in the noradrenaline excretion during cold stress A fall in the noradrenaline excretion of the reserpinized animals was usually followed by death These results suggest that reserpine treatment inhibits the synthesis of noradrenaline in vivo and that the survival of moderately cold stressed adrenalectomized animals is related to the secretion of noradrenaline

The ability of reserpine to depress the catecholamine content of various organs has been demonstrated by several investigators (CARLSSON and HILLARP 1956 HOLZBAUER and VOGT 1956 WEIL MALHERBE and BONE 1959 MUSCHOLL 1959, and others) In high concentrations reserpine directly depletes the adrenergic nerve transmitter granules of their stores of noradrenaline EULER and LISHAJKO 1960) BERTLER, HILLARP and ROSENGREN (1961) and KIRSHNER (1962) observed that reserpine blocked the uptake of dopamine by the adrenal medullary cytoplasmic granules and postulated that the synthesis of noradrenaline might be prevented through this mechanism The following experiments were designed in order to obtain information on the effect of reserpine on the synthesis of noradrenaline in adrenergic nerves in vivo They were based on the assumption that if reserpine does inhibit the synthesis of noradrenaline in vivo then a reserpinized adrenalectomized rat subjected to a cold stress should show no increase in noradrenaline excretion

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It has previously been demonstrated that exposure of rats to 2 C produces a sharp rise in noradrenaline excretion (LEDUC 1961) Previous workers have also shown that rats exposed to 2 depend to a large extent on the calorigenic properties of noradrenaline to maintain normothermia (HSIEH CARLSON and GRAY 1957 LEDUC 1961) However the role of noradrenaline in the survival of rats subjected to moderate cold is not known It was hoped that the experiments described below would help to elucidate the part played by this catecholamine

Materials and methods

All experiments were carried out on male rats of the Sprague Dawley strain purchased from Anticimex Stockholm. Rats were kept at least one week at room temperature (20 C) before being used for experimentation The weights of the rats ranged from 175 g to 250 g The animals were given a well balanced commercial brand of laboratory food They were allowed food and water ad libitum

Rats were bilaterally adrenalectomized and divided into two groups Both groups were given desoxycorticosterone acetate and cortisone acetate daily from the time of the operation Seven days after adrenalectomy the rats were placed in metabolism cages at 20 ± 1 At this point daily reserpine treatment was initiated in one group of animals The other group of rats received no reserpine and thus served as control animals After 4 days in the metabolism cages at 20 both groups of rats were transferred to 13 ± 0 5 Reserpine treatment was maintained for the one group throughout the duration of the cold exposure

Experiments were arranged to start at 10 o clock in the morning and the urine was collected for the next 24 hours Urine was obtained from individual rats in wire metabolism cages coated with plastic paint to prevent oxidation of the catecholamines by metallic ions The cages were mounted on polyethylene funnels with a plug of glass wool inserted in the neck This allowed the filtration of urine but retained feces A hydrochloric acid was added to the urine to maintain a pH of approximately 3 At the end of every 24 hour period care was taken to secure uniform collection of urine by applying slight pressure on the abdomen of the animals in order to produce reflex urination Each metabolism cage was thoroughly rinsed with dilute acid and the washings added to the urine samples

Each urine specimen was filtered and assayed for free adrenaline and noradrenaline according to the method of EULER and LISHAJKO (1961 a) Catecholamine values were expressed as μg of adrenaline or noradrenaline excreted in the urine per kilogram of body weight per 24 hours For organs rats were killed by a blow on the head and the organs immediately removed weighed and extracted with trichloracetic acid The organ extracts were then assayed for adrenaline and noradrenaline in the same manner as the urine samples The organs of four rats were pooled for extraction

Body temperature was measured by inserting a thermocouple 3—4 cm inside the rectum

Rats were injected subcutaneously with 2 mg of desoxycorticosterone acetate daily A solution of desoxycorticosterone acetate in oil 10 mg/ml was employed Cortisone acetate in an aqueous suspension of 25 mg/ml was injected intramuscularly in a dose of 2 5 mg/rat Reserpine (Serpasil Ciba) was prepared in a solution of 0 25 mg/ml and injected subcutaneously in a dose of 0 25 mg/kg/day Phenoxybenzamine (Dibenzyline Smith Kline and French) 10 mg/kg was administered intraperitoneally in a solution containing 10 mg/ml

Table I Catecholamine content of organs μg of catecholamine /g of tissue

Control		Reserpine	
Noradrenaline	Adrenaline	Noradrenaline	Adrenaline
Heart			
0 57 ± 0 10	0 06 ± 0 015	0 00 ± 0 00	0 015 ± 0 000
Spleen			
0 49 ± 0 06	0 04 ± 0 030	0 015 ± 0 014	0 02 ± 0 01
Liver			
0 03 ± 0 00	0 0035 ± 0 000	0 003 ± 0 002	0 001 ± 0 00

Results

Adrenalectomy elicited an abrupt fall in body weight in all rats The average loss was 30 g However one week after the operation all animals were able to maintain their weight The initiation of reserpine treatment at this point caused these rats to again lose weight with an average loss of 10 g per week Forty eight hours before death the reserpine treated animals usually showed a rapid drop in weight of 15 to 20 g Although the reserpine treated rats became sedated they could easily be aroused by handling

The administration of reserpine for 4 consecutive days at 20 produced a marked depression or complete depletion of the adrenaline and noradrenaline normally found in the heart spleen and liver These values accompanied by control values are given in Table I

The urinary levels of adrenaline and noradrenaline for control and reserpinized rats placed first at 20 and then at 13 are shown in Fig 1 The control rats significantly (P ~ 0 02) increased their noradrenaline excretion within the first 24 hours at 13 and maintained this for the duration of the experiment The adrenaline values in the urine showed a gradual increase All of the control animals survived the experiment and appeared in good health after 10 days of cold exposure Reserpine treated rats at 20 showed depressed urinary values of noradrenaline and adrenaline Upon exposure to 13 these rats failed to show a significant increase in the urinary excretion of noradrenaline and adrenaline

A fall in the excretion rate of noradrenaline was usually followed by the death of the animal Only 5 of the 12 reserpinized rats remained normothermic and lived following 10 days of cold exposure Although as shown in Fig 1 7 rats were recorded as living on day 10 in the cold 2 of these subsequently died within 2 hours of taking the samples One reserpinized rat showed a dramatic increase in urinary noradrenaline augmenting from 3 5 μg kg/24 hours at 20 to

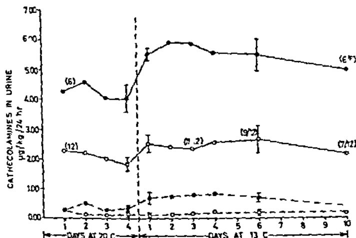


Fig. 1 Urinary excretion of catecholamines from adrenalectomized rats exposed to 20 C and 13 C. Numbers in parantheses indicate the number of animals in each group at 20 C and number of rats surviving at 13 C Standard errors are plotted on day 4 at 20 C and days 1 and 6 at 13 C

—— Amount of noradrenaline excreted. — — — Amount of adrenaline excreted. ● Control rats. ○ Reserpine treated rats

9 1 on day 4 at 13° This rise in noradrenaline was subsequently followed by a fall and death of the animal on day 10 All reserpinized rats remained normothermic to within 24 hours of death

Four of the surviving normothermic reserpine rats were given phenoxybenzamine and left at 13 C Three of the animals became hypothermic and died within 24 hours Four additional rats were adrenalectomized and left at 20 Seven days later reserpine treatment was initiated After 4 days of reserpine treatment they were placed at 13 Twenty four hours later all 4 rats appeared healthy and were normothermic These animals were then given phenoxybenzamine and within 24 hours 3 of them became hypothermic and died The fourth rat appeared unaffected by the phenoxybenzamine

Discussion

Euler and Hillarp demonstrated in 1956 that noradrenaline is largely stored in discrete cytoplasmic granules found within the nerve axon The discovery that these granules could be separated from adrenergic nerves by homogenization and high speed centrifugation has allowed some of their properties to be described (Euler 1958 Schümann 1958 Euler and Lishajko 1961 b c) In addition to acting as storage sites these particles presumably play a role in the synthesis of noradrenaline While the decarboxylation of dihydroxyphenylalanine (dopa) to dihydroxyphenylethylamine (dopamine) occurs in the

cytoplasmic sap of adrenal chromaffin cells (BLASCHKO, HAGEN and WELCH 1955) the subsequent conversion of dopamine to noradrenaline requires the presence of cytoplasmic granules (KIRSHNER 1962) Following stimulation of an adrenergic nerve the noradrenaline released is assumed to be drawn from a free pool which is subsequently replenished from the granules in response to a fall in the cytoplasmic concentration of this catecholamine (EULER and LISHAJKO 1962 cf HILLARP 1960) It has been suggested that reserpine prevents the synthesis of noradrenaline (BERTLER HILLARP and ROSENGREN 1961, KIRSHNER 1962) by blocking the uptake of dopamine by the adrenal medullary granules The results of the work presented here suggest that reserpine also inhibits the biosynthesis of noradrenaline in nerve granules

Reserpine treatment for 4 consecutive days significantly depressed or even depleted various tissues of their adrenaline and noradrenaline content This is shown in Table I Thus any significant increase in noradrenaline excretion induced by cold exposure must be a result of an increased biosynthesis The reverse is also true A failure of the cold stress to increase noradrenaline excretion most likely implies a biosynthetic block Fig 1 shows that in the reserpinized rats when placed at 13 no significant increase occurred in the noradrenaline excretion These results therefore suggest that reserpine inhibits the increase in the biosynthesis of noradrenaline normally induced by cold exposure These conclusions are in agreement with CREEN and SAWYER (1960) who suggested that reserpine impairs the synthesis of noradrenaline in rat brain tissue In contrast to the results obtained with the reserpine animals the adrenalectomized control rats significantly increased their noradrenaline excretion within the first 24 hours at 13 and maintained this level throughout the duration of the experiment

Several of the reserpinized rats showed an initial tendency to increase their urinary noradrenaline excretion This initial temporary increase might result from (1) an initial increase in biosynthesis which fails within a few days (2) the release of remaining tissue bound noradrenaline and or (3) the stimulation of extra adrenal chromaffin tissue containing noradrenaline The presence of noradrenaline in the extra adrenal chromaffin tissue of young animals has been shown (WEST SHEPHERD and HUNTER 1953 SHEPHERD and WEST 1952) It is possible that the reserpine treated rats used in this study still possessed varying amounts of noradrenaline in extra adrenal chromaffin tissue An unusually large amount of noradrenaline stored in such tissue is the most probable source of the high noradrenaline excretion values obtained in one of the reserpinized rats

LEDUC (1961) demonstrated that both intact and adrenalectomized rats placed at 2 showed an immediate increase in noradrenaline excretion The results presented in this paper indicate that even moderate cold stress (13) is sufficient to elicit a significant increase in noradrenaline excretion The control animals also showed a slight increase in the urinary excretion of adrenaline

following cold exposure This was not surprising and probably resulted from the stimulation of extra medullary chromaffin tissue It was expected that the adrenalectomized control rats would maintain homeothermia and live at 13 LEDUC (1961) showed that adrenalectomized rats maintained on the same dose of corticoids as employed in this study were able to live for long periods at 2

Previous investigators have demonstrated that both normal and cold acclimated rats depend upon an increased secretion of noradrenaline and/or adrenaline to sustain life at 2° (HSIEH *et al* 1957 LEDUC 1961) The results of the present study show that adrenalectomized rats depend upon the secretion of noradrenaline to maintain homeothermia at 13 As was stated in Results a fall in noradrenaline excretion of the reserpine treated rats was accompanied by hypothermia and death In addition 6 of the 8 normothermic reserpinized rats treated with phenoxybenzamine became hypothermic and died within 24 hours at 13 The ability of phenoxybenzamine to significantly diminish the calorigenic effect of noradrenaline has previously been demonstrated (JOHNSON and SELLERS 1961) It must therefore be concluded that the survival of adrenalectomized rats placed in a moderately cool environment is dependent upon the secretion and action of noradrenaline

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Acta physiol scand 1963 59 445—453

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Excitation of Flexor Fusimotor Neurones by Electrical Stimulation in the Red Nucleus

By

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Abstract

Appelberg B and I Z Kosary *Excitation of flexor fusimotor neurones by electrical stimulation in the red nucleus* Acta physiol scand 1963 59 445—453 — The effect of electrical stimulation at various depths in the region of the red nucleus upon the activity in fusimotor efferents or muscle spindle afferents from extensor and flexor muscles of the hind limb was studied in cats lightly anesthetized with Nembutal Stimulation in the dorsal part of and dorsally to the nucleus caused inhibition of the activity in most fusimotor fibres and decreased the static discharge of extensor as well as flexor spindles From the ventral part of the nucleus the activity in many fusimotor fibres was facilitated Fusimotor fibres receiving facilitation from the red nucleus evidently pass to flexor muscle spindles because the discharge of these was markedly increased from stimulating points in the ventral part of the red nucleus The discharge of extensor spindles was decreased also from this part of the nucleus The findings are discussed mainly in relation to previous observations of motor effects elicited from the red nucleus

In a previous investigation (Appelberg 1962) it was shown that the red nucleus exerts an inhibitory influence upon the activity in fusimotor neurones and thereby causes a decrease in the afferent discharge of extensor as well as flexor muscle spindles In 1957 it was reported by Pompeiano (cited by Dow and Moruzzi 1958) that electrical stimulation in the red nucleus in the decerebrate cat causes contralateral fore and hind limb flexion The finding of excitatory action on flexor motoneurones and inhibition of extensor neurones from the red nucleus (Sasaki Namikawa and Hashiramoto 1960) confirmed Pompeiano s experiments Furthermore Pompeiano (1957) and also Maffei and Pompeiano (1962) demonstrated that there exists within the red nucleus a somatotopical organization with regard to flexor effects exerted on fore and hind limbs This was in complete concordance with a histological demonstration

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GREEN, H. and J L. SAWYER Intracellular distribution of norepinephrine in rat brain. I Eff ct of reserpine and the monoamine oxidase inhibitors trans-2 phenylcyclopropylamine and 1 isonicotinyl 2 isopropyl hydrazine *J Pharmacol exp Ther* 1960 *129* 243—249

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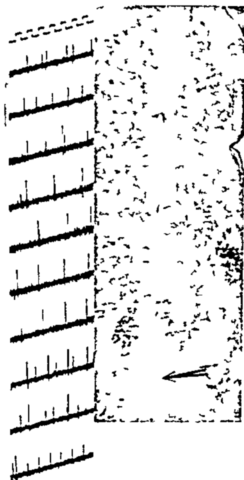


Fig 1 Left Recording from ventral root filament containing sp kes f om two fus motor neurones St mulat on in the ed nucl u du ing artifacts in th d to sev nth trace from above Right Stimulating sit mark d by electrocoagulation in ventral part of the ed nucleus (arrow) Time 20 msec

twitch. Usually the effect of electrical stimulation in the region of the red nucleus upon the activity in the filaments recorded from was testeo at intervals of 0 25 mm along the stimulating tracks Histological sections (celloidin embedded 50 μ thick and stained with toluidin blue) were used for making magnified tracings of the region of the stimu lating track through the red nucleus in each experiment Effective stimulating points were indicated on the tracings using an el ctrocoagulation made at the end of each ex periment as a reference point The shrinkage of the brains during the histological procedure was est mated by using the Horsle Clarke zero line through the aqueduct as a second referenc

Results

1 *Recordin, from ventral root fibres*

The first observation made in experiments of this type was that on stimu lation of a single point within the red nucleus different effects were obtained on

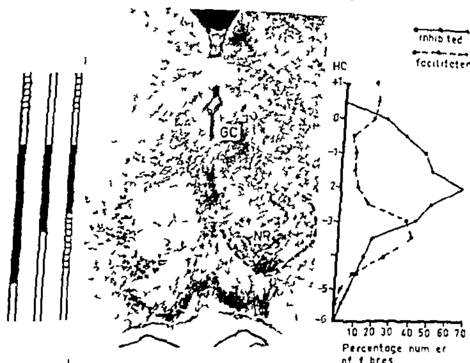


Fig 2 Diagrammatic representation of facilitatory and inhibitory effects on the activity in fusimotor fibres by electrical stimulation in the red nucleus In the photomicrograph is indicated the borderlines of the nucleus except dorsally where it is difficult to define In the diagram to the right is illustrated the percentage number of fibres inhibited or facilitated at different depths The ordinate gives the depths in Horsley Clarke coordinates To the left three common filter types black bars indicating inhibition lined part of columns facilitation Abbreviations GC Griseum centrale HC Horsley Clarke NR Nucleus ruber

the activity of different fusimotor fibres i e some fibres were inhibited and others facilitated An example of this is given in Fig 1 which shows recordings from two different fibres which were situated in the same ventral root filament The big spike had practically no spontaneous activity (mean of 4 imp sec measured over a period of 5 sec) but became activated by the rubral stimulation (to a mean of 20 imp sec) The smaller spike on the other hand had a rather high spontaneous firing 45 imp sec which however was strongly inhibited upon stimulation in the red nucleus (down to 11 imp sec) The localization of the stimulating point is shown in the photomicrograph of a histological section The electrode tip was placed in the ventral part of the red nucleus

The next interesting observation was that from certain localizations of the stimulating electrode the activity in some fusimotor neurones were inhibited at a low stimulating strength but facilitated at a higher strength By moving the

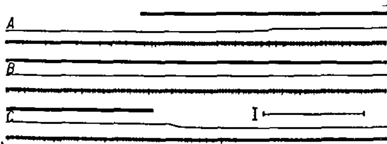


Fig 3 Recording from muscle spindle afferent from the tibialis anterior muscle A B and C are consecutive parts of the same record The thick line in each record indicates the period of stimulation in the red nucleus The myograph beam (above the spikes) shows contraction of the muscle during the stimulation period Time calibration 1 sec Myograph calibration 20 g Initial tension approximately 45 g

electrode up and down in the track it was then possible to find nearby regions yielding pure inhibition or facilitation to any stimulating intensity

In Fig 2 are summarized the results obtained by recording from fortyfour different fusimotor fibres in seven experiments The photomicrograph shows a frontal section through the red nucleus at the level of the stimulating track in one experiment In the diagram to the right is indicated the percentage of the number of fibres investigated which were inhibited (solid curve) or facilitated (dashed curve) at different stimulating depths (given along the ordinate in Horsley Clarke horizontal coordinates and corresponding to the section) It is evident from the diagram that from the dorsal part of and also dorsally to the red nucleus the majority of the fibres were inhibited From stimulating points in the ventral part of the nucleus on the other hand the main effect was facilitation although some fibres were inhibited A dorsally placed region (at the level of the aqueduct) from which some fusimotor fibres were facilitated should also be noted The columns to the left in Fig 2 represent three common types of fusimotor fibres met with in the present experiments The solid black parts of the columns indicates inhibition the lined parts facilitation

Inhibitory as well as facilitatory actions on the activity in fusimotor fibres may thus be elicited from the red nucleus in the lightly anaesthetized cat There is a clear tendency for the facilitatory effects to be obtained from electrode positions ventrally in the nucleus Inhibitory effects are mainly elicited from a region in the dorsal part of and dorsally to the red nucleus The observation that some fibres were inhibited only while others were facilitated as well indicates a different function of these two groups of fibres The experiments to be described below were designed as an attempt to find the destination of the different types of fusimotor fibres With regard to the observation mentioned above to the effect that electrical stimulation in the red nucleus causes flexion of contralateral limbs a study of flexor spindles appeared to be of special interest.

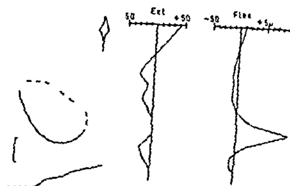


Fig 4 Diagrammatic representation of simultaneous effects upon the activity in one extensor (left graph) and one flexor (right graph) spindle evoked by electrical stimulation in the red nucleus Abscissa inhibitory (—) and facilitatory (+) effects expressed as the percentage change of the discharge frequency Ordinate corresponds to the tracing of the red nucleus to the left Scale 1 mm

2 *Recording from muscle spindle afferents from extensor and flexor muscles*

Experiments in which recording was made from thin dorsal root filaments containing single afferents from muscle spindles demonstrated that flexor spindles could be facilitated from the red nucleus Such an effect is illustrated in Fig 3 where A, B and C are consecutive parts of the same recording The thick line above the recording indicates the period during which stimulation was applied in the red nucleus The stimulus is seen to cause a considerable increase in the frequency of discharge of the spindle The myograph tracing above the spikes reveals a contraction of the tibialis anterior muscle In experiments of this type it was regularly observed that while a low stimulating strength elicited spindle activation only a stronger stimulus caused also a muscle contraction The contraction could sometimes be so vigorous as to cause unloading of the spindle and a decrement in its discharge The elicitation of the contraction was independent of the dorsal roots being intact or not This was verified in experiments in which the dorsal roots L 5 to S 1 on the side recorded from were cut

The area within the red nucleus yielding these facilitatory effects appeared to agree well with the area causing facilitation of fusimotor activity This is shown in Fig 4 where the approximate border lines of the red nucleus as well as a diagrammatic representation of simultaneous effects of rubral stimulation on the activity in one extensor (left graph) and one flexor (right graph) spindle afferent is illustrated Along the abscissa in each diagram are plotted the inhibitory (—) or facilitatory (+) effects at different depths along the track through the nucleus It is apparent from the diagrams that both spindles were inhibited from the dorsal part of the red nucleus and also from a region slightly dorsally to the nucleus From the ventral part of the nucleus on the other hand the flexor spindle was facilitated while the extensor spindle was uninfluenced The region at the level of the aqueduct yielding facilitation of the activity in both filaments should also be noted This region corresponds well to the one causing facilitation of some fusimotor fibres (cf Fig 2

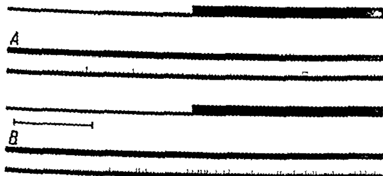


Fig 5 A Simultaneous recording from extensor (middle beam) and flexor (lower beam) spindle afferent Note inhibition of extensor spindle and facilitation of flexor spindles during stimulation in the red nucleus (broad line above)

B Same as A but recording made approximately 15 min later after injection of additional 25 mg Nembutal Note lack of facilitation of flexor spindle Time calibration 1 sec

In other experiments of this type it was observed that facilitation of flexor spindles was regularly obtained by stimulation in the ventral part of the red nucleus Extensor spindles were on the other hand only inhibited The inhibition was elicited either from the dorsal part only or from the entire track through the nucleus

In experiments with recording from muscle spindle afferents the influence of different levels of anaesthesia on the effects caused by central stimulation was also observed In such experiments it appeared that upon deepening the anaesthesia the spontaneous firing of both extensor and flexor spindles was markedly depressed The effect of central stimulation changed in such a way that in deep anaesthesia facilitatory effects on flexor spindle activity could no longer be obtained This is illustrated in Fig 5 which shows inhibition of an extensor (middle beam in A) and facilitation of a flexor (lower beam in A) spindle in light anaesthesia In B which was obtained 15 minutes later after an additional 25 mg of Nembutal had been injected intravenously facilitation of the flexor spindle could no longer be obtained while the inhibitory effect on the extensor spindle was even more marked The increased basic frequency of the flexor spindle in B is due to a slight increase of the load on the muscle

Discussion

The results described above reveal that electrical stimulation in the dorsal part of the red nucleus exerts an inhibitory influence upon fusimotor neurones to extensor as well as flexor muscle spindles in hind limbs muscles This results in a lowering of the static discharge of these spindles On the other hand the same type of stimulation in the ventral part of the nucleus exerts a reciprocal effect with inhibition of extensor spindles but facilitation of flexor spindles The facilitatory path to the spindles is more sensitive to anaesthesia than the

hibitory one This may be an explanation of the observation that upon deepening the anaesthesia the activity in afferents from muscle spindles is depressed Reciprocal effects on extensor and flexor muscle spindles were previously described by SHIMAZU HONGO and KUBOTA (1962) who stimulated the globus pallidus the internal capsule and the caudate nucleus These authors obtained the reciprocal response in deeply anaesthetized preparations while in animals under light anaesthesia only facilitation of all spindles was elicited Diffuse and reciprocal effects were thus sensitive to anaesthesia in a manner different from that seen when stimulating the red nucleus The fact, however that similar reciprocal effects may be elicited both from the above mentioned regions and from the red nucleus seems to add some proof to the theory that the red nucleus is a component in a cerebral gamma controlling system (cf APPELBERG 1962)

The observation that the dorsal border of the inhibitory region did not agree with the dorsal border of the nucleus may be explained in different ways First it should be noted that the dorsal border of the red nucleus is in most cases very difficult, not to say impossible to define in the type of histological sections used (cf photomicrograph in Fig 2) The nucleus may therefore extend further dorsally than can easily be recognized in the sections Secondly a certain spread of the stimulus may account for some of the discrepancy between effective stimulating area and anatomical struture It cannot however be excluded that the functionally inhibitory region does not coincide exactly with the area anatomically known as the red nucleus

The experiments by SASAKI, NAMIKAWA and HASHIRAMOTO (1960) previously mentioned seem to prove that the red nucleus influences the alpha motoneurones This fact alone could serve as a satisfactory explanation of the observations made by POMPEIANO and coworkers i e flexion effects elicited from the nucleus It is however now evident that stimulation in the red nucleus exerts a combined effect on alpha and fusimotor systems Stimulation of the central part of the red nucleus thus seems to excite alpha motoneurones to hind limb flexor muscles and simultaneously to elicit a contraction of the intrafusal muscle fibres in flexor spindles Inhibition of alpha as well as fusimotor neurones to extensor muscles will also result from such a stimulus The observation that week stimulation may cause spindle activation alone while stronger stimulation causes also contraction might indicate that the alpha activation is secondary to the increased discharge of the spindles — i e the alpha activation might be due to reflex effects exerted via the gamma loop Such an assumption is however contradicted by the observation that the contraction may still be elicited in de afferented preparations Alpha and fusimotor effects elicited from the red nucleus thus seem to be independent phenomena

The fact that inhibition of extensor as well as flexor spindles is obtained from the dorsal part of the red nucleus is worthy of note with respect to POMPEIANO s view of this part of the nucleus as a fore limb center The observation seems to indicate some kind of simultaneous reciprocal control of fore and hind limbs

The area at the level of the aqueduct which upon stimulation yielded activation of flexor as well as extensor spindles seems to agree with a region described by MAFFEI and POMPEIANO (1962) They reported the so called tegmental response to be elicited from this region It seems possible that the contralateral leg extension seen in this response may be the result of an increased fusimotor — and possibly also alpha — activity to extensor as well as to flexor muscles In this connection it is of interest also to consider the experiments by GRANIT and HOLMGREN (1955) These authors obtained extensor spindle activation from a region in the mesencephalic tegmentum which according to their description was situated dorsally and laterally to the red nucleus GRANIT and HOLMGREN suggested the rubrospinal or reticulospinal tracts to convey these effects It now seems likely that the latter alternative is the correct one since extensor spindle activation could never be obtained in the present experiments with rubral stimulation

The red nucleus is no doubt an important relay for central control of extra as well as intrafusal muscle tone From the experiments with stimulation in the ventral part of the red nucleus it is evident that the cells controlling alpha and fusimotor neurones are not differently localized within this part of the nucleus From a stimulating site here both effects can always be elicited On the other hand the effects have different thresholds extrafusal contraction being obtained at a higher stimulating strength than the fusimotor activation This may be of importance for future attempts to separately study the one system or the other

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Acta physiol scand 1963 59 454—461

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Effect of Adenine Nucleotides on Catecholamine Release and Uptake in Isolated Adrenergic Nerve Granules

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Abstract

Euler U S v and F Lishajko *Effect of adenine nucleotides on catecholamine release and uptake in isolated adrenergic nerve granules* Acta physiol scand 1963 59 454—461 — On addition of 1—5 mM adenosine triphosphate (ATP) the spontaneous loss of noradrenaline from a suspension of adrenergic nerve granules is retarded or prevented Adenosine diphosphate (ADP) exerts a similar effect while adenosine monophosphate (AMP) has little action ADP and ATP also enhance the uptake of catecholamines from the incubation fluid This effect is observed even with low amine concentrations in the incubation fluid in contrast to the uptake without addition of ATP The ATP-dependent uptake is inhibited by reserpine

Partially depleted noradrenaline storage granules prepared from bovine splenic nerves according to Euler and Lishajko (1963 a) are able to take up noradrenaline and other catecholamines when these are added to the incubation fluid in concentrations above 1 μg ml This uptake appears to be larger in isotonic sucrose than in phosphate buffer and increases with raising pH values from 6 to 8 5 Euler and Lishajko 1963 b) Under optimal conditions the granules after partial depletion may be replenished even up to the original content An uptake of similar kind has been described for adrenal medullary granules by Carlsson and Hillarp (1961)

Increased uptake of amines has been reported when adrenal medullary granules have been incubated with noradrenaline or adrenaline in the presence of adenosine 5 triphosphate (ATP) and Mg^{2+} (Kirshner 1962 Carlsson Hillarp and Waldeck 1962)

In the present report some effects of ATP and $MgCl_2$ on noradrenaline release rate and amine uptake in adrenergic nerve granules will be described

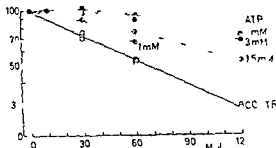


Fig 1 Noradrenaline content in adrenergic nerve granules incubated for various times in isotonic potassium phosphat at pH 7 0 and 20 C in controls and with addition of ATP in concentrations of 1—5 mM Ordinate noradrenalin in sediment after incubation in per cent of original amount Abscissa time in min

Methods

Adrenergic nerve granules were obtained in a suspension by squeezing bovine splenic nerves with 0 3 M sucrose or 0 13 M potassium phosphate at pH 7 or 7 5 as diluting fluid (EULER and LISHAJKO 1961 a) The press juice from 1 g nerve was diluted with 6–10 ml incubation fluid Larger particles when present were removed by centrifugation at about 1 000 × g for 10 min The supernatant which is free from microscopically visible cell particles contains about 1 μg free noradrenaline per ml in addition to the granule bound amine

The effect of ATP ADP and AMP on the spontaneous release of noradrenaline from granules incubated at 20 and 37 C was studied by adding the nucleotides in concentrations of 0 3—5 mM to the incubation fluid either in single or repeated additions Also the effect of ATP and $MgCl_2$ on the uptake of noradrenaline and adrenaline was measured after partial spontaneous depletion of the noradrenaline content by incubation at 20 or 37 for 60 and 10 min respectively which caused a depletion of about 50 % of the original amount After this period the amines were added to the suspension in concentrations of 0 3—20 μg/ml and the suspension incubated again usually for 30 min at 20

After the incubation period the granules were sedimented by centrifugating for 30 min at 50 000 × g resuspended in fresh isotonic K phosphate at pH 7 and recentrifugated The noradrenaline in the sediment was extracted with 1 ml 0 1 N HCl and 0 1 ml of 5 % metaphosphoric acid and estimated fluorimetrically according to EULER and LISHAJKO (1961 b)

Results

I Effect of adenosine phosphate and Mg on the spontaneous release of noradrenaline from nerve granules

When bovine splenic nerve granules are incubated at 20 in isotonic potassium phosphate pH 7 0 the noradrenaline content falls along an approximately exponential course to about 20 % of the original amount in 2 hours On addition of ATP in concentrations 1—5 mM the noradrenaline content of the granules falls off much more slowly during incubation at 20 (Fig 1) From the figure it emerges that no loss of noradrenaline occurs during incubation up to 30 min when ATP is present in concentrations of 1 5—5 mM After this time the noradrenaline content falls off more rapidly for 1 5 mM than for 3 and 5 mM ATP With a concentration of 1 mM the effect is only observed up to

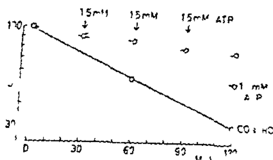


Fig. 2. Noradrenaline content in adrenergic nerve granules incubated for various times in isotonic potassium phosphate at pH 7.0 and 20° C in control and with addition of ATP 1.5 mM at zero time and each half hour. Ordinate: noradrenaline in sediment after incubation in per cent of original amount. Abscissa: time in min.

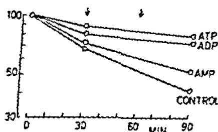


Fig. 3. Noradrenalin content in adrenergic nerve granules incubated for various times in isotonic potassium phosphate at pH 7.0 and 20° C in control and with addition of ATP, ADP and AMP 1.5 mM each half hour. Ordinate: noradrenaline in sediment after incubation in per cent of original amount. Abscissa: time in min.

30 min. After this period the release rate is the same as in the control. No effect was observed with the addition of 0.3 mM of ATP.

Since the disappearance of the effect may be due to rapid breakdown of ATP the influence of further additions of ATP each half hour was studied. As seen in Fig. 2 each addition of ATP to a concentration of 1.5 mM retarded the net loss of noradrenaline, suggesting that the ATP-concentration has to be maintained at this level in order to prevent a loss of noradrenaline from the granules.

Addition of 5 mM $MgCl_2$ to the suspension of granules before incubation at 20° causes a slight inhibition of the release rate or no effect at all. When ATP and Mg^{2+} were added together in concentrations of 5 mM to the original suspension of nerve granules the "protecting" effect was the same as with ATP alone or very slightly increased.

Since the prevention of loss of noradrenaline from the nerve granules when incubated with ATP might depend either on a blocking of the spontaneous release or on an uptake of noradrenaline concomitant with the normal spontaneous release it appeared of interest to study the effect of incubation with ATP in the presence of adrenaline. If a block had occurred as with reserpine, then adrenaline would not be expected to be taken up. However, it was found that adrenaline was taken up and noradrenaline released in about equivalent amounts. This suggests that the "protecting" effect of ATP on the endogenous noradrenaline in the absence of adrenaline is due to an uptake of this amine balancing the release (cf. Discussion).

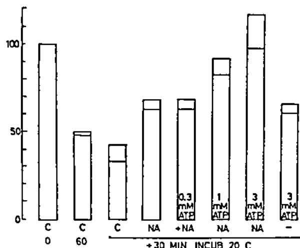


Fig 4 Noradrenaline content in adrenergic nerve granules incubated for 30 min in isotonic potassium phosphate at pH 7 0 and 20 C after 60 min preincubation at 20 Control (C) addition of noradrenaline (NA) 20 μg/ml and of adenosine triphosphate (ATP) 0 3—3 mM after partial depletion for 60 min (2 experiments) Ordinate noradrenaline in sediment in per cent of original amount

In other experiments the effect of ATP was compared with that of ADP and AMP As seen in Fig 3 addition of ADP has an effect which is similar to that of ATP while AMP had little effect

II *Effect of ATP and Mg^{2+} on the uptake of catecholamines in adrenergic nerve granules*

It was observed that the uptake of noradrenaline added to a nerve granule suspension was considerably enhanced by addition of ATP in concentrations of 1—5 mM No effect was observed with 0 3 mM ATP This effect was not increased by addition of Mg^{2+} when incubation was made in the original suspension of granules

Fig 4 shows the effect of various concentrations of ATP on the noradrenaline uptake in a partially depleted granule suspension (2 exp) As seen in the figure addition of NA alone or ATP alone causes some uptake However this is larger when 1 and 3 mM ATP is given together with NA In 3 exp when ATP was added alone the noradrenaline uptake was 13 16 and 16 per cent of the original amount This uptake of NA probably depends on the presence of the free noradrenaline which occurs in a concentration of about 1 μg/ml in the original suspension

In granules which have been sedimented and resuspended in fresh phosphate buffer solution an increased uptake of noradrenaline was also noted after addition of ATP together with the noradrenaline but it was always less than in the original suspension Addition of Mg^{2+} in these experiments increased the uptake considerably Table I shows an experiment in which the threshold of noradrenaline required for a measurable uptake in resuspended and partially depleted granules was determined An uptake was noted even with 0 3 μg/ml

Table I Uptake of noradrenaline in resuspended and partially depleted adrenergic nerve granules incubated 30 min at 20 C with ATP, Mg^{++} and noradrenaline

Pre incubation 30 min 37 (resuspended granules)	Incubation 30 min 20 with additions	ATP 3 mM Mg^{++} 5 mM added	Noradrenaline µg/ml added	Noradrenaline per cent of original in granules after incubations
–	–	–	–	100
+	–	–	–	58
+	+	–	–	47
+	+	+	–	50
+	+	+	0 3	63
+	+	+	1	67
+	+	+	3	5
+	+	+	10	77

noradrenaline in the incubation fluid added after sedimentation and resuspension in isotonic potassium phosphate pH 7 0 As seen in the table preincubation of the granules for 10 min at 37 caused a depletion of 42 % increasing to 53 % after additional incubation for 30 min at 20 Addition of ATP and Mg^{2+} alone caused no definite uptake in contrast to the experiments in which ATP was added to the original suspension containing about 1 µg ml noradrenaline In this experiment the noradrenaline concentration was maximally about 0 1 µg/ml in the incubation fluid, which presumably was too low to permit an uptake

No definite difference is observed in the uptake with 3 and 10 µg/ml of noradrenaline in the incubation fluid suggesting that the uptake mechanism is working at near maximal capacity even at 3 µg/ml (about 2 10⁵ M)

A comparison of the uptake of noradrenaline and adrenaline in various concentrations is seen in Fig 5 In the figure the uptake is measured from the level reached by previous depletion represented by the base line An appreciable noradrenaline uptake is noted when ATP is added alone (2) and of adrenaline when this amine is added alone (3) No 4—6 show the effect of the amine concentration on the uptake The greatest uptake is seen for adrenaline in no 4 and for noradrenaline in no 6 When adrenaline and noradrenaline are both added to the incubation fluid the uptake is lower than for each one of the amines given alone It is also seen that in the presence of ATP the uptake is enhanced (no 3 and 4) While there is an uptake of noradrenaline when ATP alone is added (2) hardly any uptake of this amine is noticed when adrenaline is present in a high concentration (10 µg/ml) (4) Apparently the higher concentration of adrenaline suppresses the uptake of noradrenaline when this amine is present only in about 1 µg/ml

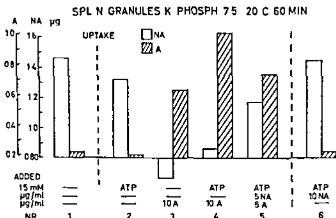


Fig 5 Horizontal line level reached by partial depletion during incubation 60 min at 20 C Original amount and uptake of amines indicated by ordinates for noradrenaline and adrenaline 1 before partial depletion 2—5 uptake from depletion level in the presence of ATP 1 5 mM No 6 uptake of noradrenaline in the presence of ATP 3 mM Ordinates μg amines per incubation sample

The ATP Mg dependent uptake is strongly inhibited by reserpine in concentrations of 1—10 μg/ml as noticed also for adrenal medullary granules by CARLSSON *et al* (1962) and by KIRSHNER (1962)

Discussion

The presence of relatively large amounts of ATP in catecholamine storage granules in the adrenal medulla (HILLARP HOGBERG and NILSON 1955 BLASCHKO *et al* 1956) and in adrenergic nerves (SCHUMANN 1958) has stimulated speculation as to its possible role It has been pointed out as especially noteworthy that the concentration of ATP (and ADP + AMP) normally appears to be such that the number of negative charges corresponds to the positive charges of the amines present Under certain conditions such as after reserpine (BURACK WEINER and HAGEN 1960) and in granules from chromaffin tumour cells (SCHUMANN 1960 HILLARP LINDQVIST and VENDSALU 1961) these proportions do not obtain

In the present report it is shown that ATP and ADP enhance the amine uptake also in nerve granules In addition it has been shown that the uptake in the presence of ATP and Mg^{2} also occurs with lower concentrations of noradrenaline than without ATP

No data seem to have been presented earlier referring to the influence of ATP on the rate of loss of catecholamines from granules This is slow in adrenal medullary granules (HILLARP and NILSON 1954) but occurs at a more rapid rate in suspensions of adrenergic nerve granules (EULER and LISHAJKO 1961 a)

Our present results show that if the concentration of ATP is maintained in the incubation medium at 1 mM or higher the spontaneous loss of noradrenaline is greatly retarded In a concentration of about 1 5 mM ATP

completely prevented the loss for at least 30 min whereafter it started at a slow but gradually increasing rate Repeated additions of ATP after periods of 30 min effectively prevented the loss for periods up to 2 hours, suggesting a relatively rapid inactivation of ATP

This finding might be compared with the effect of noradrenaline in the incubation medium as described previously (EULER and LISHAJKO 1963 a)

As to the mechanism underlying the 'protecting' effect of ATP on the noradrenaline content of the granules incubated under conditions that would otherwise lead to a considerable loss of the amine no definite statements can be made as yet Two possibilities present themselves, viz blocking of the 'spontaneous' release, or release and uptake at equal rates In view of the ready uptake of noradrenaline even in low concentrations from the incubation fluid in the presence of ATP the maintenance of the noradrenaline content in the granules may be explained by assuming an uptake balancing the spontaneous release

This assumption is supported by the observation that, on addition of adrenaline 10 μg/ml and ATP to undepleted granules the noradrenaline content falls and an approximately equivalent amount of adrenaline is taken up This suggests that noradrenaline is continuously and spontaneously released and that the available binding sites become occupied by adrenaline which is present in higher concentration In the presence of reserpine 10 μg ml no adrenaline is taken up during incubation of undepleted granules while such an uptake is observed in the absence of reserpine when adrenaline is added in concentrations of 5—20 μg/ml (EULER and LISHAJKO 1963 c) This is interpreted as a result of lack of binding sites since reserpine effectively retards the spontaneous release of noradrenaline

As regards the 'protecting' effect of exogenous noradrenaline on the amine content in the granules during incubation even without ATP Mg this might also be due to either a blocking of the release or a continuous balanced exchange (EULER and LISHAJKO 1963 a) We have observed that the noradrenaline content of granules after incubation with adrenaline is slightly but significantly smaller than in the controls This could mean that adrenaline has a releasing effect on the noradrenaline in the granules However the effect might also be explained by assuming that normally some of the released noradrenaline is taken up, whereas adrenaline if present in a higher concentration is taken up preferentially owing to a more favourable concentration gradient We are therefore inclined to believe that the 'protecting' effect of added noradrenaline as well as that of ATP is due to an uptake balancing the spontaneous release rather than to a blocking of the release This is also supported by the observation (Table I) that ATP Mg has only a small 'protecting' action on the noradrenaline in resuspended granules in a medium containing at most 0 1 μg noradrenaline per ml

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Effects from the Sensorimotor Cortex on Ascending Spinal Pathways

By

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Abstract

LUNDBERG A U NORRSELL and P VOORHOEVE *Effects from the sensorimotor cortex on ascending spinal pathways* Acta physiol scand 1963 59 462—473 — The effect of stimulation of the sensorimotor cortex on ascending spinal pathways was investigated in cats with recording of mass discharges with intracellular recording from the cell bodies and with unit recording from the axons Apart from effects caused by primary afferent depolarization there are marked effects from the sensorimotor cortex only on pathways influenced from the flexor reflex afferents (FRA) This holds true for the ventral spinocerebellar tract which is inhibited from the FRA and from cortex and for the ventral spinobulbar tract which is excited from the FRA and from cortex The same parallelism between actions from cortex and the FRA was found for the dorsal spinocerebellar tract but not for the spinocervical tract which have FRA activated neurones that are not excited from the sensorimotor cortex

Several ascending spinal pathways are influenced by the flexor reflex afferents (FRA) and it has been suggested that they carry information regarding flexor reflex patterns Detailed investigations have revealed a parallelism in inhibitory supraspinal control of transmission from the FRA to motoneurones and to the ascending spinal pathways (ECCLES and LUNDBERG 1959 HOLMQVIST and LUNDBERG 1959 HOLMQVIST LUNDBERG and OSCARSSON 1960 a) The FRA interneurones of spinal reflex arcs are effectively excited from the sensorimotor cortex (LUNDBERG and VOORHOEVE 1962 LUNDBERG NORRSELL and VOORHOEVE 1962 CARPENTER LUNDBERG and NORRSELL 1962) and it was therefore of interest to investigate the effect from cortex also on the ascending spinal pathways influenced from the FRA We have confirmed the finding that ventral spinal pathways are influenced in the same way from cortex and from the FRA (MAGNI and OSCARSSON 1961) and also examined effects on the dorsal spinocerebellar tract (DSCT) and the spinocervical tract

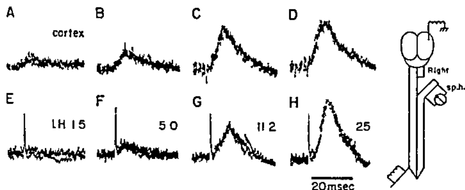


Fig 1 Ascending spinal discharge evoked from the sensorimotor cortex The recording was made from the right spinal half (except dorsal column) The right sensorimotor cortex was stimulated in A—D with 1 2 4 and 5 stimuli respectively Record E—H shows the effect of stimulation of the left hamstring nerve (1H) The strength of stimulation is given in each record in multiples of threshold strength for the VSCT discharge All records consist of superimposed traces

Methods

The experiments were made on cats under light anaesthesia from pentobarbital sodium The discharges in ascending pathways were usually recorded from dissected fasciculi or spinal halves as described by LAPORTE LUNDBERG and OSCARSSON (1956 a) but steel microelectrodes insulated except at the tip were also used For technique of intra axonal recording see LAPORTE LUNDBERG and OSCARSSON (1956 b) intracellular recording from the cell bodies of the spinocervical tract were made as described by ECCLES ECCLES and LUNDBERG (1960) The technique of cortical stimulation has been described by LUNDBERG and VOORHOEVE (1962)

Abbreviations DSCT dorsal spinocerebellar tract VSCT ventral spinocerebellar tract, bVFRT bilateral ventral flexor reflex tract FRA flexor reflex afferents ABSm anterior biceps semimembranosus nerve PBSt posterior biceps-semitendinosus nerve, G—S gastrocnemius soleus nerve FDL flexor digitorum longus nerve SP superficial peroneal nerve (deprived of muscle branches DP deep peroneal nerve (deprived of cutaneous branches)

Results

Bilateral Ventral Flexor Reflex Tract (bVFRT)

Fig 1 illustrates that on stimulation of the sensorimotor cortex there is a discharge in the dissected spinal half (stimulating and recording arrangements shown in the diagram of Fig 1) In record A a single cortical stimulus evokes a small discharge B C and D show the effects of 2 4 and 5 stimuli respectively In record F—H the contralateral hamstring nerve was stimulated in F at the minimal strength giving a maximal VSCT discharge in F—H at the stronger strength indicated in each record With separate recording from the ventral and the dorsal quadrant of the spinal half it was found that the discharge from cortex was evoked in pathways ascending in the ventral quadrant Unit recording from axons revealed excitation from cortex of the bVFRT (bilateral ventral

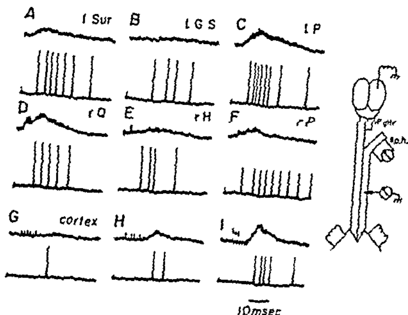


Fig 2 Cortical effect on a bVFRT unit Lower traces are recording from axons and the upper traces are the mass discharges recorded from the dissected right spinal half (except dorsal column) Record A—F show the effect of volleys in the indicated left and right nerves The right sensorimotor cortex was stimulated in G—I at increasing strength The abbreviations denote l Sur left sural nerve l GS left gastrocnemius-soleus nerve lP left peroneal nerve rQ right quadriceps nerve rH right hamstring nerve rP right peroneal nerve

flexor reflex tract) This is a spinobulbar tract activated by volleys in the FRA from a bilateral receptive field (OSCARSSON 1958 LUNDBERG and OSCARSSON 1962) 12 units of this type were found and 11 of them were effectively activated from cortical stimulation Fig 2)

Ventral Spinocerebellar Tract (VSCT)

Effects on the ventral spinocerebellar tract (VSCT) was investigated by employing the monosynaptic test discharge evoked by Ib volleys from the contralateral hamstring nerve (OSCARSSON 1957 1959 ECCLES HUBBARD and OSCARSSON 1961) Fig 3 record A shows the test discharge and B the inhibitory effect by a volley in the contralateral sural nerve Record C shows that cortical stimulation also inhibits the VSCT (MAGNI and OSCARSSON 1961) In D cortex was stimulated at the same strength as in C but the testing Ib volley from the hamstring nerve was increased to bring out the test VSCT discharge to a height slightly above that in A Record E shows that when the conditioning volley in the sural nerve now is added it gives a more effective inhibition than in B Since there is spatial facilitation in the inhibitory paths from the FRA and from cortex it can be postulated that there is convergence to the same inhibitory interneurones from the two sources Unit recording was made from 11 VSCT units Two of them were inhibited from cortex without any preceding

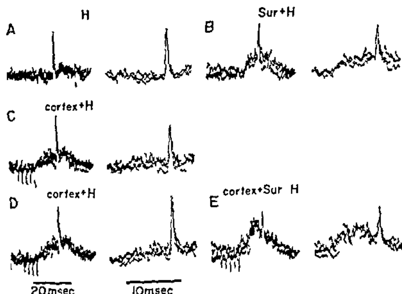


Fig ? Effect from cortex on the VSCT As in Fig 1 recording from the right spinal half and stimulation of peripheral nerves on the left side and of the right sensorimotor cortex. The test VSCT discharge (record A) is inhibited by a conditioning volley in the sural nerve (B) and by cortical stimulation (C). In D and E the test was increased and E shows the effect of combined stimulation of cortex and the sural nerve. The abbreviations denote H hamstring nerve Sur sural nerve

excitation. The remaining unit was facilitated from cortex despite the fact that the same unit received inhibitory action from the FRA. The explanation for the facilitation is probably activation of the more frontal cortical area from which VSCT can be facilitated (MAGNI and OSCARSSON 1961)

Dorsal Spinocerebellar Tract (DSCT)

Effects on the DSCT were investigated exclusively by unit recording from the tract. DSCT axons were not identified by antidromic activation from the cerebellar cortex. However, it is known that units receiving monosynaptic activation from group I muscle afferent belong to the DSCT (LUNDBERG and OSCARSSON 1960). The effect of cortical stimulation was tested on the resting discharge or on transmission of group I volleys at threshold for activation of the cells. Twenty two units activated exclusively by group I afferents were tested. None of them could be excited from the contralateral sensorimotor cortex. Slight inhibition of transmission was found in 4 of these units. Facilitation was found in 3 and the remaining 15 were not influenced from cortex. In sharp contrast to these neurones was another group of DSCT neurones receiving excitation from group I muscle afferents but which in addition were influenced by the FRA. LUNDBERG and OSCARSSON (1960) have recently

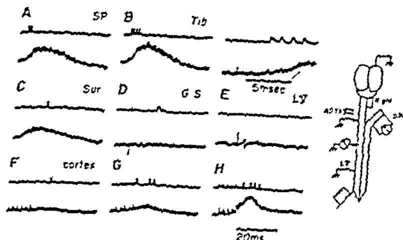


Fig 4 Effect from cortex on DSCT neurone activated from group I afferents and from the FRA. Upper traces are unit recording from an axon in the lateral funicle and the lower traces are mass discharges recorded from the dissected right spinal half (cf diagram). The nerves stimulated in record A—D are indicated. E shows the failure to activate this axon by stimulation of the lateral funiculus in L5. F—H illustrate the effect of cortical stimulation at increasing strength. The left and right traces in record B were taken simultaneously at different speeds. Record A, B (left trace), C, F, G and H were taken at the slow speed. Record D and E and the right trace in B were taken at the fast speed. The abbreviations denote SP superficial peroneal nerve (deprived of muscle branches), Tib tibial nerve, Sur sural nerve, G—S gastrocnemius-soleus nerve.

groups of this type those receiving excitation and those receiving inhibition from the FRA. Both these groups are strongly influenced from cortex, the former group excited and the latter inhibited. Three group I activated units received excitation from the FRA and could also be excited from the sensorimotor cortex as is illustrated in Fig. 4 in F—H. This unit had a monosynaptic connection from group I afferents of the G—S nerve (record D). Excitation from the superficial peroneal nerve and from the sural nerve is seen in A and C respectively and from the mixed tibial nerve in B. Three group I activated DSCT neurones were found to receive inhibition from the FRA and they could all be inhibited from cortex. This is illustrated in Fig. 5 for a neurone monosynaptically activated from the DP nerve. The resting discharge (10 superimposed traces) is shown in G and H. A strong conditioning volley in the nerve to Q gives the inhibition in A and a volley in the sural nerve the inhibition in B. Records D—F show that the effect from G—S was elicited from high threshold afferents. Inhibition of the resting discharge during a similar period was obtained on stimulation of the sensorimotor cortex (record C).

Another group of DSCT neurones is known to receive excitatory action from the FRA without having any monosynaptic connections from group I afferents. In order to identify these units as belonging to DSCT it is desirable to employ antidromic stimulation from the cerebellar cortex because the spinocervical tract (cf. next section) has a subdivision which is also activated by the FRA. It was

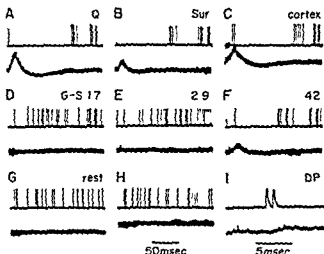


Fig. 5. Cortical effect on group I activated DSCT neurone receiving inhibition from the FRA. Recording and stimulation arrangements are the same as in Fig. 4. Record I at fast speed shows the monosynaptic activation from group I afferents of the DP nerve. The unit had a resting activity as is shown in G and H and volleys in the FRA inhibit the resting discharge (A, B and F). In D—F the G—S nerve is stimulated at the strength indicated which is in multiples of threshold strength for the VSCT discharge recording in the contralateral dissected spinal half. The inhibition in C was obtained by a train of 6 stimuli to the right sensorimotor cortex. A—H consist of 10 superimposed traces. The abbreviations denote the following left hindlimb nerves: Q, quadriceps nerve; Sur, sural nerve; G—S, gastrocnemius soleus nerve; DP, deep peroneal nerve.

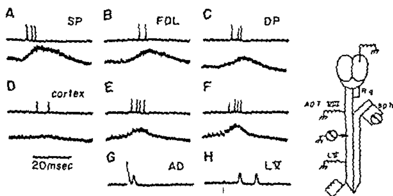


Fig. 6. Eff t f om cortex on FRA act vated neuron as umed to belong to DSCT. Recording and stimulation arrangements are the same as in Fig. 4. The rec pu e t d of this neurone is ips lateral and A—C sho the discharges evoked from the periph ral ne es ind cated. The sensorim tor co tex was st mulated at ncreas ng st ngth in D—F. G show the antidromic response and in H st mulation of the lateral fun culus in L5 does no exc te the urone but g es a synapt cally transm tted discharge. The abbreviations den te: SP, superf cial peroneal nerv ; FDL, flexor d g torum longus nerv ; DP, deep peroneal nerve (d ved of cutaneous bran hes); AD, ant drom c stimulation.

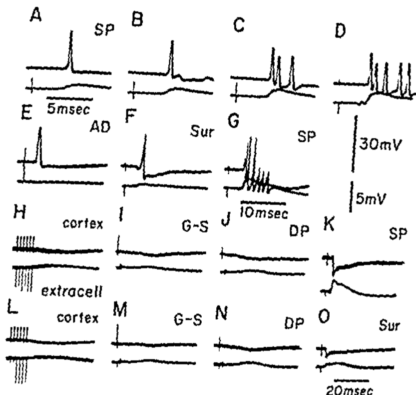


Fig 7 Intracellular recording (upper traces) from a spin cervical tract neurone Recor l ng was made from the dorsal horn in L7 with a microelectrode filled with 3M KCl The lower traces are triphasic records from the L7 dorsal root entry zon E hows th antidromic r ponse evoked from the intact dor olateral funicle 10 5 cm rostral to the site of the m croel ctrod record ng The neurone receives effective excitatory action f om the SP nerve (A—D and J at slower speed) and weaker effect from the sural nerve (F There is no effect on stimulation f the G—S an l DP nerves (E and J) and cortical stimulation is al ineffect ve (H) I ecord K—O were ob tained after withdrawal of the microelectrode to a just extracellula posit n P c rds A—F were taken at the lo and records F—O at the high ampl ficat on and cat d Fer rec ds A—F he time calibration is shown below A for F and G below record G and f r H O bel w recor l O The abbreviations denote SP superficial per neal n rve (deprived f muscle l ranches) S r ural nerve G—S gastrocnemius-soleus nerve DP d ep peroneal nerve depriv l f cutaneou branches)

BERG and OSCARSSON 1961) were able to distinguish units of this tract from the DSCT subdivision discu ed above becau e the cells of origin were located below the caudal level of CLARKE s column and hence the axons could be ac tivated by a stimulus applied to the lateral funiculus in L5 During the present investigation we found 6 units activated exclusively by the FRA with axons that could not be stimulated in L3 Five of these units were effectively activated from cortex as is illustrated in Fig 6 It is likely that they belong to the DSCT and not to the spinocervical tract because similar units identified as belongin to the spinocervical tract did not receive the corresponding effe tive excitatory action from cortex cf below

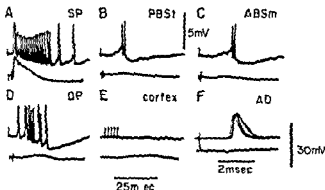


Fig 8 As in Fig 7 but intracellular recording from a spinocervical tract neurone activated from the FRA Record F shows the antidromic response evoked from the intact dorsolateral funicle 10 5 cm rostral to the site of microelectrode recording A—D show the effect of volleys in the indicated peripheral nerves There is no effect on stimulation of cortex (record E) Records A—E were taken at the higher amplification and F at the lower The abbreviations denote SP superficial peroneal nerve (deprived of muscle branches) PBSt posterior biceps-semitendinosus nerve ABSm anterior biceps-semimembranosus nerve DP deep peroneal nerve (deprived of muscle branches) AD antidromic stimulation

The spinocervical tract

The spinocervical tract already discussed in the preceding section is located in the most dorsomedial part of the lateral funicle (LUNDBERG and OSCARSSON 1961) The cell bodies are located in the dorsal horn close to the entry zone of the primary afferents (ECCLES ECCLES and LUNDBERG 1960) and the axons terminate in the upper cervical segments (LUNDBERG and NORRSELL unpublished) It has two subdivisions 1) Neurones activated exclusively by low threshold cutaneous afferents and adequately by light stimuli from very small receptive fields 2) Neurones activated by volleys in the FRA and adequately not only by light stimuli from a relative small skin field but also by stronger stimuli from a larger receptive field Twenty six units of the former type were investigated In 23 of them there was no excitation on cortical stimulation nor could the discharge be facilitated from cortex In the remaining 3 units strong cortical stimulation evoked one spike Intracellular recording from the cell bodies was made from 4 neurones of this type and gave similar results as is illustrated in Fig 7 This neurone was most effectively activated from the superficial peroneal nerve (A—D at increasing strength of stimulation and G at slower sweep speed) but received excitation also from the sural nerve (record F) Record H—J shows that stimulation of cortex (—S and DI has no effect (compare corresponding extracellular record in L—N)

Similar findings were made with the spinocervical tract neurones which receive excitation from the FRA Twelve units of this type were found and none of them received effective activation from cortex Seven of these units could not be activated from cortex and 5 received weak excitatory action at stronger

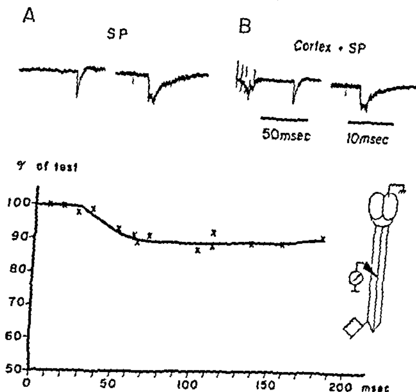


Fig 9 Inhibitory effect from cortex on transmission to the spinocervical tract Recording was made in L1 from the most dorsomedial part of the lateral funicle with a steel microelectrode insulated except at the tip Record A shows the discharge (at two sweep speeds) evoked by a volley in the superficial peroneal nerve and in B the sensorimotor cortex was stimulated as well The time course of the inhibition is shown in the graph Time interval is between the first cortical stimulus and the incoming SP volley Records A and B consist of superimposed traces

ical stimulation In addition intracellular recording was made from the cell bodies of 2 neurones of this type and cortical stimulation had no effect in any of them as is illustrated in Fig 8 This cell was very effectively activated from the superficial peroneal nerve and also received excitation from high threshold afferents in the nerves to PBSt, ABSm and DP (B C and D) but cortical stimulation did not evoke an EPSP (record E) The differences in cortical effect on FRA activated neurones of the DSCT and the spinocervical tract could be due to the different segmental levels from which these tracts take origin and not be indicative of a functional difference in the supraspinal control of the two tracts This possibility cannot be excluded but there was at least evidence in the present experiments of effective activation from cortex of FRA interneurones adjacent to the cells of the spinocervical tract in the dorsal horn of L7 as has been reported separately by LUNDBERG NORRSELL and VOORHOEVE (1962)

It was occasionally found that cortical stimulation could give a slight inhibition of transmission from cutaneous afferents to spinocervical tract neurones

This is illustrated in Fig 9 in which the discharge in the spinocervical tract was recorded with a needle electrode in the most dorsomedial part of the lateral funicle Cortical stimulation inhibits this discharge slightly (record B) and the time course of the inhibition is shown in the graph The long duration of the inhibition suggests presynaptic inhibition and such an action would be ex pected since primary afferent depolarization of cutaneous afferents can be evoked from the sensorimotor cortex (ANDERSEN ECCLES and SEARS 1962 CARPENTER LUNDBERG and NORRSELL 1962)

Discussion

It has been postulated that ascending spinal pathways influenced by the FRA may carry information regarding flexor reflex patterns (ECCLES and LUND BERG 1959 LUNDBERG 1959, HOLMQVIST LUNDBERG and OSCARSSON 1960 a MAGNI and OSCARSSON 1961) This could be achieved if the interneurones transmitting effects from the FRA to motoneurones and to ascending pathways were influenced in a similar way not only from the periphery but also from higher centres Since the FRA interneurones of reflex arcs can be effectively activated from the sensorimotor cortex (LUNDBERG and VOORHOEVE 1962 LUNDBERG NORRSELL and VOORHOEVE 1962) it has now been possible to test this hypothesis further by investigating effects from cortex on different ascending spinal pathways activated from the FRA

Apart from effects due to primary afferent depolarization there are marked effects from cortex only on ascending pathways influenced from the FRA These effects are evoked from cortical areas at a strength of stimulation that gives action to reflex arcs via the pyramidal tract It is assumed that also the present effects are mediated by the pyramidal tract (cf MAGNI and OSCARSSON 1961)

We have confirmed the finding of MAGNI and OSCARSSON (1961) that the VSCT is inhibited and the bVFRT excited from cortex and agree with their interpretation that the effects are exerted through interneurones transmitting actions from the FRA HAGBARTH and KERR (1954) reported that cortical stimulation inhibited transmission from cutaneous afferents to a ventral path way This may be occlusion or else it could be due to primary afferent depo larization of the FRA (cf ECCLES KOSTYUK and SCHMIDT 1962)

The similarity between action from cortex and from the FRA on the DSCT is also striking The neurones which do not receive monosynaptic excitation from group I muscle afferents but which are strongly activated from the FRA are very effectively excited from the sensorimotor cortex Of the group I ac tivated DSCT neurones only those receive action which in addition are in fluenced by the FRA and there is inhibition from cortex if the FRA inhibits and excitation if the FRA excites It is postulated that also the effects on the DSCT are exerted through excitation from cortex of interneurones transmitt actions from the FRA

By contrast the spinocervical tract is little influenced from the sensorimotor cortex The slight longlasting inhibition of monosynaptic transmission to this tract is in all likelihood presynaptic and would be expected since primary afferent depolarization in cutaneous afferents can be evoked from the sensorimotor cortex (ANDERSEN ECCLES and SEARS 1962 CARPENTER LUNDBERG and NORRSELL 1962) However there is no activation from cortex of the neurones which are excited exclusively from low threshold cutaneous afferents and no or only weak excitatory action on the other main group of neurones which in addition is activated from the FRA

Both types of spinocervical tract neurones carry information through MORIN s (1955) path to the somato-sensory cerebral cortex (NORRSELL and VOORHOEVE 1962 ANDERSSON 1962 NORRSELL and WOLTON unpublished) and it is of interest that there is no positive feed back from cortex Previous investigations have shown that the direct spinocerebellar tracts are never activated from the cerebellar cortex although other ascending spinal pathways may be strongly activated (LUNDBERG and OSCARSSON 1960 1961 1962 HOLMQVIST LUNDBERG and OSCARSSON 1960b) However absence of positive feed back to ascending spinal tracts from the terminal centre may not be a general rule cf JABBUR and TOWE 1961 GORDON and JUKES 1962)

In the monkey KUYPERS (1960) has distinguished between the sensory and motor projections of the pyramidal tract There is evidence of projection to the spinal cord from the sensory cortex but the parallel activation of interneurones transmitting effects from the FRA to ascending pathways and to motoneurones makes it likely that the now described effects on ascending tracts is a function of the motor cortex The difference in cortical effects on the FRA activated neurones of DSCT and the spinocervical tract is of special interest The FRA information of the DSCT may like that of the VSCT and bVFRT signal interneuronal activation of reflex arcs The FRA information of the spinocervical tract on the other hand may be more directly sensory However with many of these DSCT and spinocervical tract neurones the FRA activation may be subsidiary to the strong monosynaptic effect from cutaneous afferents

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Time Correlation Between the Effects of Reserpine on Behaviour and Storage Mechanism for Arylalkylamines

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Abstract

CARLSSON A J JONASSON and L ROSENGREN *Time correlation between the effects of reserpine on behaviour and storage mechanism for arylalkyl amines* Acta physiol scand 1963 59 474—477 — The time course of the effect of reserpine on the ability of the adrenal medullary granules to take up arylalkylamines in vivo has been studied It was observed that there was a closer correlation in time between the effects of the drug on the uptake of the amines by the granules and behaviour than between the latter and the tissue amine content The finding is in accordance with a hypothesis that new formed arylalkylamines are at first transferred to sites in the granules where they are easily available for physiological release

Some years ago the effect of reserpine was proposed to be due to its ability to deplete 5 hydroxytryptamine and catecholamines from body tissues (SHORE, SILVER and BRODIE 1955 BERTLER CARLSSON and ROSENGREN 1956) The effect of the drug on the amines was determined by measuring the total tissue levels It has however been observed by HILLARP (1960) that the tissue catecholamines are stored in different pools — one labile and one much larger inert fraction These different pools should be taken into account in attempts to analyse the effects of drugs (cf CARLSSON 1963) In this work the in vivo effect of reserpine on the two fractions has been studied and compared to the effect on the behaviour of the animals

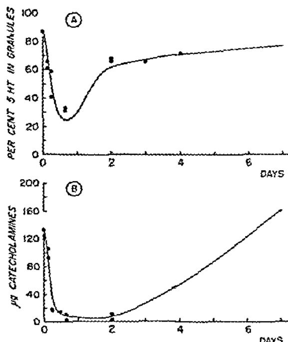


Fig 1 A. Uptake of 5 hydroxytryptamine by the adrenal medullary granules after 5-HTP administration various times after reserpine. *Ordinate* 5 hydroxytryptamine in the granules in per cent of the total amount found in the adrenal medulla Fig 1 B Catecholamines in the adrenals of the rabbit various times after reserpine

Methods

Male adult rabbits weighing about 2 kg were used throughout the experiments In order to estimate the efficiency of the adrenal medullary granules to take up amines a method previously described was employed (BERTLER HILLARP and ROSENGREN 1960) The animals were given 2 mg kg of reserpine After various intervals the animals received 75 mg/kg of DL 5 hydroxytryptophan (5 HTP) intravenously One hour after the injection of 5 HTP the animals were killed by air embolism The adrenal medulla was freed from the cortex on an ice chilled glass disk The granules were prepared as described by BERTLER *et al* 1960 The 5 hydroxytryptamine contents of the granules and the cytoplasmic sap were determined after purification on a column containing Amberlite XE 64 (BERTLER and ROSENGREN 1959) The results are given in Fig 1 In another series of experiments the catecholamines of the adrenals were determined at various time intervals after administration of reserpine to the animals (2 mg kg) The results are found in Fig 1

Results

As will appear from Fig 1 reserpine interfered with the uptake of 5 hydroxytryptamine into the storage granules The effect was observed to occur already 3 hours after the reserpine injection and reached its maximum within 16 hou The ability of the granules to take up the amine was restored to two third the normal value on the second day after reserpine administration an rose more slowly on the following days

The catecholamine content of the adrenals gradually decreased during the first few hours after the reserpine injection. It reached its minimum within one or two days. Then it began to rise only slowly. It was fully restored after about one week.

The pharmacological actions of reserpine appeared 15—20 min after the injection. They were almost completely vanished after 36 hours. At this interval the ability of the adrenal medullary granules to take up the monoamine was largely restored, whereas their catecholamine content was still at minimum.

Discussion

It is now generally accepted that the pharmacologic actions of reserpine are mostly mediated through its interference with the storage mechanism for tissue catecholamines and 5-hydroxytryptamine. There are, however, some observations which seem to argue against such a view. Thus, after a single injection of a large dose of reserpine the pharmacologic effects disappear within the first two days, whereas the concentrations of tissue catecholamines and 5-hydroxytryptamine still remain at their lowest points (Fig. 1; see also CARLSSON et al. 1957). Another objection has been that repeated administration of small doses of reserpine may reduce the catecholamine contents of the tissues to only a few per cent of the normal values (HAGGENDAL and LINDQVIST 1963), but in spite of this, the animals may exhibit no obvious actions of the drug.

The last mentioned observations indicate that the actual content of amines of the tissues is of only minor importance for the function. As shown by HILLARP (1960) the amines are stored in different fractions. It is likely that in the major fraction of the store uptake and release occur slowly. It may be that this part mainly serves as a store from which supplies of amine may be drawn when needed. The amines of the smaller labile fraction may be more easily available for physiological release by nerve activity. In further support of the existence of two pools it may be quoted that following repeated administration of 5-HTP (or DOPA), amines accumulate but slowly as compared with the rapid accumulation following the first injection, suggesting rapid saturation of the smaller labile pool (unpublished experiments).

The present experiments are in agreement with this view. The time course of the ability of the granules to take up amines from the cytoplasmic sap after reserpine administration is more closely related to the pharmacologic effects than the actual level of adrenaline. The reason why we have studied the uptake of 5-hydroxytryptamine instead of catecholamines is purely technical: the former amine is not transformed into other amines in the adrenal medullary cell.

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Acta physiol scand 1963 59 478—485

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The Serum Cholesterol Level and Mucosal Mast Cell Count in the Gastrointestinal Tract of Rats in Dexamethasone-shock

By

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Abstract

HAIKONEN M and T RASANEN *The serum cholesterol level and mucosal mast cell count in the gastrointestinal tract of rats in dexamethasone shock* Acta physiol scand 1963 *59* 478—485 — Rats were injected with large daily doses of dexamethasone for 5 and 8 days during which they and the control rats which were not given dexamethasone were kept on a fat rich and fat poor diet The serum cholesterol was determined and the mast cells in the mucosa of the stomach duodenum and colon were counted at the end of the test period The serum cholesterol level rose distinctly in the rats given fat and it was raised significantly further by dexamethasone administration The elevation of the serum cholesterol level was small in the rats kept on a fat poor diet despite the dexamethasone administered The mast cells in the mucosa of the glandular stomach duodenum and colon were degranulated during dexamethasone treatment most rapidly in the mucosa of the glandular stomach The diet had no effect on the mucosal mast cells It is suggested that a factor activating the clearing factor is mobilised into the organism from the mucosal mast cells in the gastrointestinal tract and that this activating factor is absent after mast cell degranulation resulting in alimentary cholesterolemia

The serum cholesterol level of rats seems to be 19—72 mg per 100 *ml* (BYERS and FRIEDMAN 1960 SEIFTER *et al* 1959) Its level in the serum is obviously influenced by the kinetics of cholesterol metabolism which causes

changes Cholesterol in the diet causes elevation of the serum cholesterol concentration if the food contains in addition to cholesterol fat which dissolves it More profuse dissolution of cholesterol in the dietary fat caused a greater elevation of the serum cholesterol level since absorption obviously increased (WILKENS DE WIT and BRONTE STEWART 1962) A 2 per cent cholesterol content in the diet caused a distinct elevation of the serum cholesterol level this was however inhibited by fat free food with the same cholesterol content in the food (WILKENS and DE WIT 1962)

The surface area of the intestine used for resorption also affects the resorption of dietary cholesterol Jejuno-colonic shunt caused a drop in serum cholesterol and beta lipids in human blood (LEWIS TURNBULL and PAGE 1962) Cholesterol is released from the organism in bile in which the level is increased by the cholesterol resorbed from the food This resorption raised the cholesterol level of the organism only when it exceeded the amount of cholesterol used in bile acid synthesis (WILSON 1962) More cholesterol seemed to be consumed in the synthesis of bile acids in man than was absorbed into the organism from food (BERGSTROM 1959)

Endogenous cholesterol was synthetised in liver cells but cholesterol synthesis also occurred in fibroblasts especially in the lungs (DOUGHERTY and BERLINER 1959) More cholesterol was synthetised in the adrenal cortex than used in the production of steroid hormones (HECHTER 1958) The cholesterol synthesis of the endothelial cells of the blood vessels was accelerated when the blood pressure fell (WERTHESSEN 1959) but this was possibly accompanied by increased secretion of adrenalin and noradrenalin The last mentioned substances acted like a stress in provoking lipolysis of the tissues and lipemia (LEITES 1962) The serum cholesterol level of rats was also raised by Triton WR 1339 which slows down the movement of lipids from the blood into the tissues (BYERS and FRIEDMAN 1960)

Serum cholesterol and beta lipids are lowered by polysaccharide isolated from duodenal mucosa (BIANCHINI 1958 b) It is related chemically to heparin (BIANCHINI 1958 a) and it activates the plasma clearing factor in the manner of heparin

The polysaccharides present in the mucosa of the gastrointestinal tract are probably bound in mast cell granules These are degranulated in the gastric mucosa of rat under the influence of ACTH and glucocorticoids (RASANEN 1960) Mast cells are degranulated fairly completely throughout the rat gastrointestinal tract by dexamethasone in a short time (RASANEN 1962) As stress ACTH and glucocorticoids inactivate the serum clearing factor and at the same time raise the plasma cholesterol level (SEIFTER *et al* 1959) and on the other hand deplete the heparinoid stores of the gastrointestinal tract we decided to ascertain the extent to which dietary cholesterol affects the serum cholesterol level in rats during dexamethasone therapy

Method

Male rats aged 4—5 months of Dawley Sprague strain were used for the investigation The rats were caged in groups during the experiment and were given food and tap water ad libitum The individual consumption of food and water was not measured Dexamethasone (Decadron Merck) was injected intramuscularly the dose was 2 × 0 8 mg daily for 5 days for a part of the rats 1 × 0 8 mg was injected daily for a further 3 days i e they were treated for 8 days in all The rats were given a fat rich and fat poor diet for 3 days prior to the dexamethasone injections and the same diet was continued throughout the treatment

The *fat rich diet* consisted of 500 g of salted butter 400 g of eggs and 300 g of soft cheese The *fat poor diet* consisted of dried Finnish rye bread containing salt The composition of the diet was as follows

	Fat rich diet per cent	Fat poor diet per cent
Fat	45	2 0
Cholesterol	0 3	—
Protein	11	11 0
Carbohydrates	1 2	74
Water	41	10

The experimental groups were distributed in the following way

Fat rich diet and dexamethasone for 5 days were given to 13 rats which weighed 125—245 g when sacrificed

Fat rich diet and dexamethasone for 8 days were given to 6 rats weight 105—110 g

Fat rich diet alone was given for 11 days to 12 rats weight 120—130 g

Fat poor diet and dexamethasone for 5 days were given to 17 rats weight 11— 305 g

Fat poor diet and dexamethasone for 8 days were given to 6 rats weight 90—100 g

Fat poor diet for 11 days was given to 19 rats weight 125—160 g

The blood was collected from the animals by opening the thoracic aorta under light ether narcosis The rats given dexamethasone were killed 14—16 hours after the last injection The blood collected was centrifuged within 1 hour of taking the sample and the plasma if no signs of hemolysis were present was stored in a refrigerator for analysis The serum cholesterol level was determined by a modification of Bloor's method based on the Liebermann Burchard reaction

From the stomach duodenum and colon samples were taken immediately after the sacrifice The samples were fixed in fresh 4 per cent basic lead acetate The mast cells were counted from 10 μ mucosal sections stained with toluidine blue Their cut surface was at right angles to the surface of the mucosa Mast cells were counted from the mucosa at the height of greatest density and from 20 visual fields in the direction of the mucosa. In the stomach mast cells were most numerous in the superficial part of the mucosa in the intestinal mucosa at its mean height Magnification × 915 and oil immersion were used

Results

Diarrhea occurred in the rats receiving dexamethasone especially in those on a fat rich diet of which 5 rats died in the 5 day series before the end of the experiment In the 8-day series half of the rats given a fat rich diet and dexamethasone died Of the rats given a fat poor diet and dexamethasone 1 rat

Table I The serum cholesterol level mg per 100 ml of blood and the number of mucosal mast cells per 20 visual fields in rats after dexamethasone treatment and in controls during a fat rich and fat-poor diet

	Fat rich diet						Fat poor diet					
	Time days											
	5		8		11		5		8		11	
	Treatment											
	Dexamethasone				Controls		Dexamethasone				Controls	
	n=		n=		n=		n=		n=		n=	
Cholesterol	8	244 ± 27	2	437 ± 1 [illegible]	8	103 ± 7	8	123 ± 21	2	93 ± 1 4	[illegible]	0 ± 4 9
Mast cells												
Stomach	4	2.2 ± 1 3	2	0	12	103 ± 21	7	3 1 ± 0 6	4	0	19	99 ± 18 9
Duodenum		48 ± 1[illegible]		0		109 ± 14		36 ± 11		0		111 ± 12 1
Colon		16 ± 9.3		0.5		[illegible] ± 11		3 6 ± 1 0		0		80 ± 8 9

died in the 5-day and 3 rats in the 8-day series At the end of the experiment the rats receiving dexamethasone were tired had lost weight and their hair was yellowish and tangled Their blood coagulated rapidly on sampling and the animals kept on a fat rich diet had manifest lipemia The liver of the rats given dexamethasone was yellow

The *serum cholesterol levels* are shown in Table I The level rose distinctly ($P < 0\ 01$) in the course of 5 days in rats given dexamethasone and kept on a fat rich diet It was higher ($P < 0\ 01$) than in the rats given dexamethasone and a fat poor diet The highest serum cholesterol values were recorded in 2 rats surviving to the end of the experiment which were kept on a fat rich diet and received dexamethasone for 8 days No significant increases were observed in rats given dexamethasone and a fat poor diet although their cholesterol levels were higher than those of the control rats A fat rich diet alone seemed to elevate the serum cholesterol concentration The difference from the cholesterol values of rats kept on a fat poor diet was significant ($P < 0\ 01$)

The *mucosal mast cells* of the gastrointestinal tract were degranulated (Table I) during glucocorticoid therapy The process was more rapid in the gastric mucosa than in the intestinal mucosa but within 8 days was almost total throughout the gastrointestinal tract mucosa The nature of the diet did n[illegible] appear to have a distinct effect on the mucosal mast cells

The number of cholesterol and mast cells analyses is smaller than the number of rats with which the experiment was started The reason for this was that some of the rats died during the dexamethasone treatment the amount of blood obtained was too small for analysis or hemolysis was present Histological specimens were not taken from all the test animals or fixation failed in some of the samples

Discussion

Considerable esterification occurs in the intestinal mucosa when cholesterol is resorbed (HELLMAN and ROSEFELT 1959) Alimentary cholesterol raises the serum cholesterol level in about 2 days but it is eliminated from the organism slowly chiefly through the bowels Dietary fats appear to be necessary for the resorption of cholesterol Alimentary cholesterol may raise the serum cholesterol of rats 40 per cent in the course of 12 days (WILKENS and DE WIT 1962)

During dexamethasone treatment the serum cholesterol level seems to rise rapidly and higher than the values of the control animals on a fat rich diet the elevation in 5 days was about 125 per cent and in 8 days was even higher Serum lipemia obviously increased concurrently although no analysis was performed

The cholesterolemia of rats given dexamethasone and kept on a fat rich diet may be partly caused by an increase in the absorption capacity of the intestine like that established in Addison s disease under the combined effect of glucocorticoid and deoxycorticosterone (GUARINI MACALUSO and PAPPALARDO 1962) Moreover the mobilisation of lipids during glucocorticoid influence (LEITES 1962) may increase the blood cholesterol Furthermore glucocorticoids destroy the cells of the reticuloendothelial system from which especially from the fibroblasts (DOUGHERTY and BERLINER 1959) cholesterol may be released or its deposition in the fibroblasts be inhibited However the effect that the endogenous cholesterol synthetised in the adrenals and released from elsewhere exerts on serum cholesterol appeared to be small in the present work as the serum cholesterol level rose only slightly in the rats given a fat poor diet and dexamethasone

Polysaccharides isolated from gastric mucosa (ROSSI and RALLI 1958 CANTONE RULLI and ROSSI 1958) and duodenal mucosa (BIANCHINI 1958) have a reducing effect on the serum cholesterol and beta lipids destroy plasma microchyls and activate the clearing factor in the same way as heparin apparently by separating the lipid from the protein component BENAS KELLER BACOKA and BUCAR 1962) Mucosal polysaccharides are probably located in the mast cells of the gastrointestinal tract which are degranulated in the gastric mucosa during the glucocorticoid action (RÄSÄNEN 1960 TOLEY and CTIK 1962) and though more slowly in the intestinal mucosa (RÄSÄNEN 1962) The same phenomenon was established in the present study and the increasing

effect of alimentary fat on serum cholesterol seemed to be distinctly intensified in connection with mucosal mast cell degranulation This intensification is generally difficult to elicit in rats (WILSON 1962)

The clearing factor seems to become insufficient during dexamethasone treatment The probable reason is that there is insufficient mobilisable heparin after the degranulation of the mucosal mast cells It is possibly because of this that the plasma of these rats was markedly lipemic after a fat rich diet Heparinoid of the duodenal mucosa has been found to inhibit alimentary cholesterolemia in rats (NIGROSINI *et al* 1961)

Beta lipoproteins decrease in human blood under the influence of compound 48/80 a histamine liberator while heparinemia increases (SICUTERI *et al* 1961) Lipemia and cholesterolemia increase in rats during acute stress (SEIFTER *et al* 1959) when the mobilised glucocorticoids possibly use the polysaccharides of the organism for their conjugation After adrenalectomy during stress the elevation of the lipoid and cholesterol levels in rat blood is inhibited (SEIFTER *et al* 1959) and the degranulation of gastric mucosal mast cells also disappears under the influence of ACTH (RASANEN 1961)

Cortisone administered to rabbits for 35 days caused a drop in the plasma cholesterol although they were on a high cholesterol diet But when the rabbits were given labelled cholesterol in food after cortisone therapy the serum labelled cholesterol level rapidly rose above the precortisone value (DURY and SWELL 1960)

The anticoagulative effect of gastric secretion increases during histamine and insulin stimulation (BODI WIRTS and TOCANTIN 1956) Achlorhydric gastric juice contains fewer polysaccharides than normal or hyperacid gastric juice (GALLETTI INESI and LODI 1958) Lowered excretion of hydrochloric acid is established in human subjects with cardiac arteriosclerotic symptoms (MARKS *et al* 1962) MARKS and his co workers sought a partial correlation between the clearing reaction and gastric acidity and lipase activity A high mast cell count in human gastric mucosa (RASANEN 1958) which corresponds to a 3 000 fold blood heparin concentration may however be of significance in the clearing reaction

A fat rich diet increases the excretion of bile acids in the synthesis of which cholesterol is consumed (WILSON 1962) During dexamethasone treatment cholesterol absorption may be decreased by severe diarrhea but the concentration of cholesterol in the serum still rises

Mucosal polysaccharides of the gastrointestinal tract probably enter the blood during the absorption process As the polysaccharides are possibly located in the mucosal mast cells from which they are obviously consumed during glucocorticoid influence the clearing factor effect is inhibited and alimentary lipemia follows

Duodenal heparinoid stimulates biliary secretion and counters the inhibi effect of cholesterol on cell metabolism The activity of many enzymes decr

in rabbit tissues under the influence of cholesterol but returned or rose when duodenal heparinoid was administered (NICROSINI et al 1961) The slowing down of cholesterol intermediary metabolism in the absence of heparin or heparinoid of gut in rats given dexamethasone may also be a reason for the deposition of cholesterol in the blood

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Acta physiol scand 1963 59 486—492

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Liver Catalase Activity during Complete Extra-Hepatic Biliary Stasis in Rabbits and Dogs

By

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Abstract

ARONSEN K F and B HAEGER ARONSEN *Liver catalase activity during complete extra hepatic biliary stasis in rabbits and dogs* Acta physiol. scand 1963 *59* 486—492 — The liver catalase activity has been studied in rabbits and dogs during and after different periods of complete extra hepatic biliary obstruction In both animals biliary stasis caused a decrease in enzyme activity This decrease was most pronounced in the rabbits After release of the biliary obstruction in the dogs the reduced activity of the liver catalase returned to normal level indicating a reversible phenomenon The species difference and the cause of the decrease of the catalase activity in association with biliary stasis has been discussed

Biliary stasis affects several functions of the liver The severity of the effect depends on the degree and duration of the stasis

The hem proteid catalase a peroxidatic enzyme with a life cycle of about 10 days in guinea pigs (THEORELL *et al* 1951) occurs in relatively large amounts in the liver It was therefore thought that also the activity of this enzyme might be influenced by biliary stasis The purpose of this investigation in rabbits and dogs was to check this possibility and to ascertain whether the alteration if any was reversible.

Material and methods

Thirteen rabbits (4 males, 9 females) weighing between 3.0—3.6 kg and 13 mongrel dogs (5 males, 8 females) weighing between 7.0—16.0 kg were used for the experiments.

Rabbits

The catalase activity in the liver was studied after 1 day's complete biliary stasis in 5 animals (no. 1—5), after 2 days' stasis in 2 (no. 6—7) and after 3 days' stasis in 6 (no. 8—13).

Immediately after removal of the livers of no. 8, 9, 12 and 13, biopsy specimens were taken for histological examination.

Dogs

The normal catalase activity in the liver was determined in all 13 dogs. In 2 (no. 12—13) of them, the activity was re-examined after 3 days' complete stasis, in 5 animals (no. 1—5) after 7 days' stasis and in 5 (no. 6—10) after 14 days of stasis. At the end of the periods of stasis liver specimens were taken from dogs no. 3, 4, 7 and 8 for histological examination. 4 animals (no. 3, 5, 8 and 9) were re-examined for catalase activity in the liver 58, 30, 83, and 50 days, respectively, after release of the biliary obstruction.

In all animals in which biliary stasis was produced, the glutamic pyruvic acid transaminase (GPT) activity in the serum was determined before stasis and at the end of stasis (after 7 days in no. 1—5 and after 14 days in no. 6—10).

Anesthesia

Rabbits and dogs deprived of food for about 12 hours were anesthetized with Nembutal (Abbott) in doses of 20—25 mg per kg of bodyweight.

Operative technique

In the rabbits the common bile duct was approached by a midline incision, ligated and divided. The operative technique applied for the occlusion of the common bile duct and subsequent release of the biliary obstruction in dogs has been described elsewhere (ARONSEN 1961). In order to obtain representative liver samples, pieces of 15—20 g were removed.

Determination of catalase activity

Immediately after removal, the liver slices for determination of the catalase activity were kept in the dark at about — 20° C until they were analyzed, which was always within 1 week. After the liver samples had been thawed for 1 hour at room temperature they were washed and homogenized according to DOUNCE and SHANEWISE (1950). The mean wet weight per millilitre of the homogenates from rabbits and dogs was 0.99 g (0.94—1.01) and the corresponding mean weight after drying at + 105° C during 12 hours was 0.071 g (0.043—0.099). For determining the catalase activity, each homogenate of rabbit liver was diluted 1 : 25 (4 %), 1 : 50 (2 %), 1 : 100 (1 %) and 1 : 250 (0.4 %) and of dog liver 1 : 100 (1 %), 1 : 200 (0.5 %) and 1 : 500 (0.2 %) with distilled water.

The catalase activity in the various dilutions was determined by the method of FEINSTEIN (1949). As for the controls, each dilution was analyzed twice and the mean was calculated. The activity was expressed as millimoles of sodium perborate ($NaBO_3$) decomposed by 0.5 ml of homogenate dilution under the conditions of the method used. The calculations were made after correcting the wet weights of the original homogenates to 1.00 g per ml.

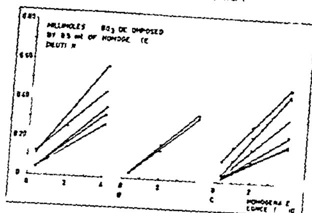


Fig 1 $NaBO_3$ decomposition related to concentration of liver homogenate in rabbits with complete biliary obstruction.

A 5 rabbits after 1 day's biliary stasis
B 2 rabbits after 2 days biliary stasis
C 6 rabbits after 3 days biliary stasis

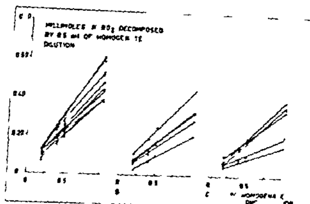


Fig 2 NaBO decomposition related to concentration of liver homogenate in normal dogs and in dogs with complete biliary obstruction

A. 13 normal dogs
B 5 dogs after 7 days biliary stasis
C. 5 dogs after 14 days biliary stasis

Determination of GPT

The GPT was determined by the method of REITMAN and FRANKEL (1957) and the results are given in Karmen units (KARMEN *et al* 1955

Histological method

Immediately after removal the biopsy specimens were fixed in 10 formalin dehydrated in alcohol and embedded in paraffin The specimens were cut in sections about 5 μ thick, which were then stained with hematoxylin-eosin

Results

Rabbits

The catalase activity found in the liver from rabbits after 1 2 and 3 days stasis are given in Fig 1 A B and C respectively The 3 groups comprised 5 2 and 6 animals The number of millimoles of $NaBO_3$ decomposed by 0 5 ml of 4 $\%_0$ homogenates ranged between 0 57 and 0 27 in group A 0 34 and 0 33 in group B and 0 49 and 0 19 in group C The amount of $NaBO_3$ decomposed in

Table I Liver catalase and GPT activity in 5 dogs before (columns I) and immediately after (columns II) 7 days total biliary obstruction The difference between pre and postoperative values are given in columns III

	mMoles $NaBO_3$ decomposed by 0.5 ml of homogenate dilution 1 100			GPT (Karmen units)		
Dog no	I	II	III	I	II	III
1	0.41	0.45	+ 0.04	41	810	+ 769
2	0.59	0.29	− 0.30	154	750	+ 596
3	0.38	0.21	− 0.17	13	1 590	+ 1 577
4	0.38	0.33	− 0.05	41	205	+ 164
5	0.43	0.33	− 0.10	58	62	+ 4
Mean value			− 0.12			+ 622

Table II Liver catalase and GPT activity in 5 dogs before (columns I) and immediately after (columns II) 14 days total biliary obstruction The difference between pre and postoperative values are given in columns III

	mMoles $NaBO_3$ decomposed by 0.5 ml of homogenate dilution 1 100			GPT (Karmen units)		
Dog no	I	II	III	I	II	III
6	0.48	0.16	− 0.32	29	735	+ 706
7	0.44	0.22	− 0.22	27	39	+ 12
8	0.52	0.37	− 0.15	69	1,230	+ 1 161
9	0.61	0.40	− 0.21	37	1 560	+ 1 523
10	0.53	0.35	− 0.18	93	970	+ 877
Mean value			− 0.22			+ 856

liver tissue from normal rabbits under corresponding conditions is between 0.71—0.48 (HAEGER ARONSEN 1962) It is thus clear that the catalase activity fell below normal in 4 of 5 animals after 1 day's stasis in 2 of 2 animals after 2 days stasis and in 5 of 6 animals after 3 days stasis

Microscopical appearance of the liver The pathological changes in the liver specimens examined were rather pronounced Thus after 3 days biliary stasis liver cell necrosis with absence of nuclei and hyalinization were almost always constant findings in different parts of the specimens In addition, leukocyte infiltration was observed in a few areas Otherwise the liver parenchyma was well preserved No bile cylinders but bile lakes were occasionally seen Further proliferation of bile ductules was more or less mark

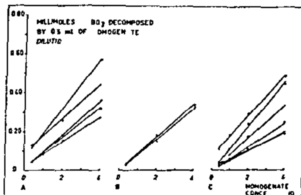


Fig 1 $NaBO_3$ decomposition related to concentration of liver homogenate in rabbits with complete biliary obstruction.

A 5 rabbits after 1 day s biliary stasis
B 2 rabbits after 2 days biliary stasis
C 6 rabbits after 3 days biliary stasis

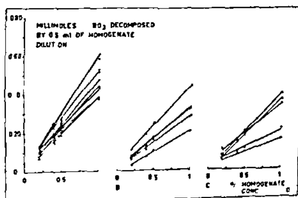


Fig 2 $NaBO_3$ decomposition related to concentration of liver homogenate in normal dogs and in dogs with complete biliary obstruction

A 13 normal dogs
B 5 dogs after 7 days biliary stasis
C 5 dogs after 14 days biliary stasis

Determination of GPT

The GPT was determined by the method of REITMAN and FRANKEL (1957) and the results are given in Karmen units (KARMEN *et al* 1955)

Histological method

Immediately after removal the biopsy specimens were fixed in 10 % formalin dehydrated in alcohol and embedded in paraffin The specimens were cut in sections about 5 μ thick, which were then stained with hematoxylin-eosin

Results

Rabbits

The catalase activity found in the liver from rabbits after 1 2 and 3 days stasis are given in Fig 1 A B and C respectively The 3 groups comprised 5 2 and 6 animals The number of millimoles of $NaBO_3$ decomposed by 0 5 ml of 4 % homogenates ranged between 0 57 and 0 27 in group A 0 34 and 0 33 in group B and 0 49 and 0 19 in group C The amount of $NaBO_3$ decomposed in

pigments were seen in the Kupffer cells Atrophy of the liver cells bile duct and canaliculi dilatation proliferation of bile ducts vascular obstruction or fibrosis were never seen

Comments

The catalase activity in the liver fell below normal value in 11 of 13 rabbits after a 1—3 day period of complete extra hepatic biliary obstruction The frequency of decreased activity was equally large in the group of animals in which biliary flow was obstructed for 1 day as in the group in which it was obstructed for 3 days The liver catalase activity was also found to be markedly decreased in 9 of 12 dogs studied A decrease was found in the groups of dogs in which biliary stasis had been induced for 7 and 14 days respectively but not after 3 days complete obstruction The decrease was most marked after 14 days of stasis The catalase activity in dogs which is normally higher per unit of weight of liver tissue than in rabbits decreases slower in the presence of biliary stasis than in rabbits This might possibly be explained by the relatively higher flow rate of bile in rabbits than in dogs (CAMERON and OAKLEY 1932)

The decrease of catalase activity in the liver during biliary stasis may be due to 1) increased release (breakdown) of liver catalase 2) inhibition of the enzyme synthesis 3) inhibited catalytic effect of the enzyme or 4) decreased amount of metabolic mass per unit of weight of liver tissue Since the weights per unit of volume of liver homogenate were the same before and after biliary stasis no substantial change can have occurred in the amount of fluid in the liver parenchyma The histological examinations of the liver preparations from dogs with biliary stasis showed no parenchymal changes of such an extent as to suggest that the 4th possibility was of any significance for the decrease in catalase activity This possibility must however be considered for the rabbits in which these histological changes were more pronounced Liver cell damage had probably occurred in both the rabbits and the dogs This was indicated by the increase in GPT during biliary stasis in the dogs This damage had probably affected the liver catalase activity by 1 of the first 3 possibilities above

The return of the catalase activity to normal in dogs after release of 7 and 14 days stasis is in agreement with the results of liver function tests reported by ARONSEN (1961)

We thank Associate Professor G Nordén Department of Pathology General Hospital of Malmo for doing the histological examination and Mrs MARIANN NILSSON for technical assistance The investigation was supported by grants from *Svenska livförsäkringsbolags nämnd för medicinsk forskning*

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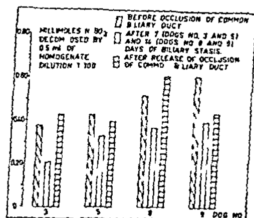


Fig 3 Catalase activity in 4 dogs normally (obliquely shaded columns) after 7 (no 3 and 5) and 14 days (no 8 and 9) total extra hepatic biliary stasis (hollow columns) and after release of stasis (horizontally shaded column)

Dogs

The catalase activity found in pieces of the liver in the dogs before and 7 and 14 days after complete extra hepatic biliary stasis are given in Fig 2 A B and C respectively The groups consisted of 13 5 and 5 dogs The number of millimoles of $NaBO_3$ decomposed by 0.5 ml of 1 % homogenates ranged between 0 61 and 0 38 before stasis and between 0 45 and 0 21 and 0 40 and 0 16 in the last 2 groups The 2 dogs (no 12 and 13) in which the common duct was occluded for 3 days showed no decrease of catalase activity in the liver The differences between pre and postoperative liver catalase activity in animals no 1—10 are given in Table I and II After 7 days biliary stasis (Table I) the number of millimoles of $NaBO_3$ decomposed by 0.5 ml of 1 % homogenates decreased on the average by 0 12 while the corresponding value after 14 days stasis was 0 22 (Table II)

On control of 4 animals (no 3 5 8 and 9) 4—12 weeks after release of the biliary obstruction the catalase activity in the liver again increased in all of the animals (Fig 3) In no 3 and 8 it had exceeded the preoperative value in no 5 it was normal while the liver catalase activity in animal no 9 was still substantially below normal 50 days after release of stasis

The GPT increased markedly after 7 days as well as 14 days stasis (Table I and II)

Microscopical appearance of the liver The pathological changes were rather few and varied in degree and extent with the duration of biliary stasis Thus the number and the size of bile thrombi in different parts of the acini progressively increased with the time of biliary obstruction Almost all the liver cells had abundant cytoplasm with distinct granules, but occasionally swollen pale-staining liver cells were observed in different parts of the specimens indicating occurrence of feathery degeneration Small leukocyte infiltrations and bile pigments in parts of the specimens with sparse liver tissue occasionally seen after 14 days biliary stasis might indicate necrosis of single liver cells and the occurrence of bile lakes. Further in specimens with bile thrombi in the canaliculi bile

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The Biological Activity of Prostaglandin E_1, E_2 and E_3

By

S. Bergstrom and U. S. v. Euler

The smooth muscle stimulating activity present in human seminal plasma and sheep vesicular glands, discovered by Euler and by Goldblatt in 1933—34 (*cf* Euler 1936) has recently been shown to be due to a series of related compounds prostaglandin E_1 E_2 E_3, $F_{1\alpha}$, $F_{2\alpha}$ and $F_{3\alpha}$ with structures shown in Fig 1 (for earlier literature see Bergstrom *et al* 1962 a and b Abrahamsson *et al* 1962 Samuelsson 1963)

The biological activity of the first isolated compounds PGE_1 and $PGF_{1\alpha}$ has been reported earlier by Bergstrom *et al* (1959 a) and the effect in human has also been studied (Bergstrom *et al* 1959 b)

We now wish to report a study of the relative biological activity of the three E-compounds that was done in connection with their isolation 1961 (Bergstrom *et al* 1962 a b) The compounds which contain a cyclopentanone structure and possess one two and three double bonds respectively have been tested on the isolated rabbit jejunum, the guinea pig ileum and on the rabbit s blood pressure The results are presented in Table I and Fig 2

When tested on the rabbit jejunum the relative activity was highest for PGE_2 and lowest for PGE_3 (Fig 2) On the rabbit blood pressure however PGE_1 was more active than PGE_2 The activity ratio PGE_1 PGE_3 was approximately the same for these two test preparations while it differed by a factor of 4—10 for PGE_1 PGE_2 on the two tests This difference may be utilized for estimating the approximate proportion of PGE_2 in a mixture of the three compounds

The same compounds have also been supplied to Dr E W Horton for a recently reported similar study (Horton and Main 1963)

When compared with the biological unit used previously it was found that 1 μg PGE_1 was equivalent to 0 2—0 25 units of a total extract of prostaglandin from human seminal fluid (HSF—PG) when tested on the rabbit jejunum and to 0 3—0 5 units on the rabbit s blood pressure (Bygdeman and Eliasson 1963) while it corresponded to 1 6 units on the guinea pig ileum

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We wish to thank mrs. I Pettersson for skilful assistance

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Catecholamines and Adenosine Triphosphate in Isolated Adrenergic Nerve Granules

By

U S v Euler, F Lishajko and L. Stjärne

The intraaxonal structures storing the sympathetic neurotransmitter (Euler and Hillarp 1956) have been shown to contain adenosine triphosphate (ATP) in an amount giving a molar catecholamine (CA) to ATP ratio similar to that found in isolated adrenal medullary granules (Schumann 1958)

The present investigation was intended to study the quantitative relationship between the CA and ATP in nerve granules during spontaneous release of amines on incubation at 37 C *in vitro*

The granules from bovine splenic nerves were isolated according to the method of Euler (1958) and suspended in ice-cold neutral isotonic potassium phosphate 5—10 ml per g nerve Coarser tissue particles were removed by centrifugation at 600—1 000 × g for 10 min at 0° C and the supernatant granule suspension was incubated for 5—30 min at 37 C. After the incubation period the suspension was centrifuged at 50 000 × g for 30 min at 0° C. The supernatant was decanted the sediment extracted with perchloric acid and the tubes were again centrifuged at 50 000 × g for 10 min The supernatant was decanted and brought to pH 5—6 with potassium carbonate An aliquot of the extract was used for fluorimetric assay of noradrenaline The rest was immediately tested for ATP using a modification of the luciferase method (Strehler and Totter 1954 p 341)

The resting content in the nerve granules of CA and ATP were found to be 13.0 ± 1.3 and 2.9 ± 0.3 mμmoles per g nerve (wet weight) respectively The molar ratio CA/ATP was 4.5 ± 0.3

On incubation at 37 C the CA content of the granules rapidly decreased whereas the ATP level was reduced much more slowly leading to a striking fall in the molar ratio CA ATP (Table I and Fig 1)

The present results demonstrate a relative constancy of the resting amount of CA and ATP in the nerve storage granules Although our values were considerably higher than those reported by Schumann (1958) the resting ratio CA ATP is of the same order of magnitude as that found by him and similar to that found for adrenal medullary granules (Falck Hillarp and Hogberg 1956 Blaschko et al 1956) However whereas adrenal granules reported to lose CA and ATP at the same rate at 37 C *in vitro*

Prostaglandin E_1 11α 15-dihydroxy-9-keto-prost-13-enoic acid

Prostaglandin E_2 11α 15-dihydroxy-9-keto-prost-5 13-dienoic acid

Prostaglandin E_3 11α 15-dihydroxy-9-keto-prosta-5 13 17-trienoic acid

Fig 1

Fig 2 Left rabbit jejunum Right unanesthetized rabbit s blood pressure From above time recording minutes blood pressure heart rate

Table I Relative activities per unit weight of PGE_1 PGE_2 and PGE_3 on the isolated rabbit jejunum the blood pressure of the unanesthetized rabbit and on the isolated guinea pig ileum (PGE_1 = 1)

	PGE_1	PGE_2	PGE_3
Rabbit jejunum	1	2 5 3 3 5	0 4 0 4 0 5
Rabbit B P	1	0 45 0 6	0 25 0 4
Guinea pig ileum	1	0 4 1 3	0 1 0 ?

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Table I Catecholamines ATP and ratio catecholamines to ATP during resting and an incubation at 37 C for different time periods N = number of observations Mean values and range (7 expts)

N	Time min	Amines mμ moles/g	ATP mμmoles/g	Ratio amines/ATP
7	0	13 0 (11 9—15 8)	2 9 (2 6—3 4)	4 5 (4 0—4 8)
1	5	9 6	2 4	4 0
4	10	7 6 (6 5—8 5)	2 5 (2 2—2 9)	3 1 (2 8—3 4)
2	15	5 5 (4 4—6 6)	2 1 (1 7—2 4)	2 7 (1 8—3 8)
2	20	3 8 (3 6—3 9)	2 1 (1 9—2 3)	1 8 (1 6—2 0)
1	30	1 8	1 9	1 0

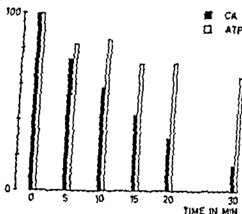


Fig 1 Catecholamines and ATP in nerve granules incubated at 37 C for different time periods Average values from 7 expts Ordinate Per cent of starting levels

an essentially unchanged ratio (Hillarp 1958) our results show that the nerve granules upon incubation lose catecholamines at a more rapid rate than the ATP leading to a striking reduction of the CA/ATP ratio This difference is another example of what may be regarded as a functional differentiation between the two types of granules

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